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EXTREME HYBRID GROWTH, GENOMIC IMPRINTING, THE LARGE X EFFECT, AND  
THE DRIVERS OF SPECIATION IN MAMMALS

By

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BS Ecology and Evolutionary Biology, University of Rochester, Rochester, NY, 2009

Dissertation

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Extreme hybrid growth, genomic imprinting, the large X effect, and the drivers of speciation in mammals

Chairperson: Jeffrey Good

Mammalian hybrids often show abnormal growth, indicating that developmental inviability may play an important role in mammalian speciation. Yet it is unclear if this recurrent phenotype reflects a common genetic basis. Here I describe patterns of hybrid inviability between two closely related species of dwarf hamsters, *Phodopus campbelli* and *P. sungorus*. Using genetic crosses, I found extreme parent-of-origin dependent growth in hybrid embryos and placentas. Abnormal growth in hybrid mammals has been empirically linked to genomic imprinting, the parent-specific silencing of a single allele that occurs in many genes involved in regulating embryonic growth. Epigenetic disruptions of genomic imprinting activate transcription of the normally silenced allele and are thought to increase expression level. Higher expression of genes whose imprinting is disrupted may cause a dosage imbalance between growth factors and repressors, ultimately leading to abnormal embryonic growth. I next tested the general prediction that disrupted imprinting leads to an increased expression of growth promoting genes in large F1 hybrid hamsters from the genus *Phodopus*. I found that disrupted imprinting correlates strongly with placental growth and changes in the expression level of imprinted genes, but that widespread disruptions in the silencing of maternally-expressed genes associates with lower, not higher, gene expression. As maternally expressed genes tend to repress offspring growth, these data suggest



that overgrowth is associated with a reduced level of growth repressors rather than an excess of growth factors.

Asymmetric hybrid phenotypes imply a genetic basis that is uniparentally inherited, for example the X chromosome, mitochondria, and imprinted genes. Hybrid dwarf hamsters in the genus *Phodopus* exhibit extreme parent-of-origin growth of both placenta and embryos. Finally, I used a suite of genetic and genomic experiments test whether the X chromosome, the mitochondria, or imprinted genes are involved in parent-of-origin dependent growth in hybrid dwarf hamsters. I demonstrated a major role for the maternally inherited X chromosome, and widespread disruptions of expression of autosomal genes including imprinted genes but no influence of the mitochondria. My data suggest that an incompatible interaction involving the maternally inherited *P. sungorus* X chromosome and a paternally inherited *P. campbelli* autosomal element results in placental and embryonic overgrowth. Overgrowth is also correlated with a greatly reduced expression of maternally-expressed imprinted genes, though any connection between expression and the X chromosome remains unclear.

I dedicate this dissertation to Kris.

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*“Are certain developmental processes especially likely to be disrupted in hybrids?  
This question has been surprisingly neglected given that hybrid defects provide a  
rare window on those developmental processes and pathways that diverge rapidly  
between taxa”*

- Coyne and Orr (2004)

Understanding the processes that generate species is a central goal in the field of evolutionary biology. In sexually reproducing taxa, new species are the result of reproductive barriers arising between different populations. These barriers can either prevent the formation of hybrids (extrinsic barriers) or reduce the fitness of the hybrids once they are conceived (intrinsic barriers). Considerable insights into general patterns of intrinsic reproductive isolation have been documented (Coyne and Orr 2004) including the identification of specific genes linked to reduced hybrid fitness (Presgraves 2010). However, one area that has remained relatively unexplored is the developmental basis of hybrid inviability (Coyne and Orr 2004).

Research into patterns of speciation has brought to light three general patterns of intrinsic reproductive isolation. First, intrinsic incompatibilities often result from deleterious interactions between divergent genes. This model requires at least two interacting loci and was put forth by Bateson (1909), Dobzhansky (1936), and Muller (1942). The Dobzhansky-Muller Incompatibility (DMI) model states that two interacting loci, which have diverged in different populations have never been evolutionarily tested and may therefore be incompatible with each other in an F1 hybrid. The DMI model has been widely accepted as the predominant mechanism by which hybrid incompatibilities evolve (Orr 1996). The two remaining patterns of reproductive isolation are so widely followed that they have been dubbed the “Two Rules of Speciation”

(Coyne and Orr 1989). These are Haldane's rule and the large X effect. Haldane's rule states that the sex with two different sex chromosomes (the heterogametic sex) is more often afflicted by deleterious interactions than the homogametic sex (Haldane 1922). Haldane's rule is followed by nearly every taxa that has chromosomal sex determination and is likely due in part to the exposure of deleterious recessive interactions that reside on the sex chromosomes (Coyne and Orr 2004). A primary explanation of Haldane's rule and the second of the "Two Rules" is the large X effect, which is the observation that the X chromosome is disproportionately involved in incompatibilities (Coyne and Orr 1989). Supporting evidence comes from *Drosophila*, where Dobzhansky (1936) found that in crosses between two races of *D. pseudoobscura* (now *D. pseudoobscura* and *D. persimilis*), the X chromosome played the largest role causing reduced testis size. Orr (1987) reaffirmed these findings that hybrid sterility was due to the *D. persimilis* X chromosome. Many recent studies in mice also support the large X effect (White et al. 2011; White et al. 2012). It is clear that many genes linked to postzygotic isolation map to the X chromosome (Coyne and Orr 2004). Both of these two rules of speciation imply that there is something unique about the sex chromosomes that result in their involvement in reproductive isolation. Moreover, they imply that the evolution of reproductive isolation may have a common genetic or developmental basis.

Along with these general patterns, many specific genes and interactions have been identified that are involved in reproductive isolation. In fact, specific genes leading to hybrid sterility or inviability have been described in *Drosophila* (*Ovd* (Phadnis and Orr 2009), *Zhr* (Sawamura and Yamamoto 1993), *Hmr* (Orr et al. 1997), and *Lhr* (Brideau et al. 2006)) and mice (*Prdm9* (Mihola et al. 2009)). However the identification of specific genes fails to encompass the entire story of speciation, as Coyne and Orr describe above. While this work has eloquently

characterized the function of these genes, it still remains unclear whether they have any general developmental trends in common. Indeed, very little is known about which specific developmental time points and pathways are affected by genetic incompatibilities (Coyne and Orr 2004).

Addressing uncertainties about whether and in what way development is involved in hybrid sterility and inviability is important to further understand the process of speciation. In terms of sterility, the failure of meiotic sex chromosome inactivation (MSCI) has long been predicted to lead to sterility in hybrid males (Lifschytz and Lindsley 1972) and recent advances indicate that MSCI failure does indeed underlie hybrid male sterility in mice (Mihola et al. 2009). However, much less progress has been made in discovering which specific developmental pathways are involved in hybrid inviability. In fact, it is unclear whether we should even expect a single pathway to play a recurrent role in inviability as developmental processes acting early in embryogenesis are often widely conserved and functionally important and therefore not likely to differ between closely related species (Coyne and Orr 2004). However, rapid changes in the expression patterns of genes involved in development are thought to be one of the main drivers of biodiversity (Carroll 2008; Brawand et al. 2011; Bolker 2000). A prime example of this is the changes in expression patterns of genes controlling beak shape in Darwin's finches (Abzhanov et al. 2004). This and other similar studies (Mallarino et al. 2012; Cohn and Tickle 1999; Shapiro et al. 2004) show that development can evolve quite rapidly, and thus may play an important role in the evolution of intrinsic reproductive isolation.

Though there may be no general developmental process that breaks down commonly across all animals (Coyne and Orr 2004), such a commonality may exist within more restricted taxonomic groups. Mammals are an excellent taxon to study the evolution of development in the

context of speciation due to the great phenotypic and morphological disparity present between relatively few species. Furthermore, many mammal hybrids show abnormal patterns of growth (Gray 1972). F1 hybrids show a huge range of sizes where some are much larger than their parents and some much smaller. Some mammalian hybrids even show parent-of-origin growth, where one hybrid is larger than the parents while the reciprocal hybrid is smaller (Allen et al. 1993; Table 1; Dawson 1965; Sokolov and Vasil'eva 1993). Parent-of-origin dependence indicates that sex chromosomes or maternal effects are involved and abnormal patterns of growth, implying that factors affecting development are disrupted in these mammalian hybrids. Intriguingly, these recurrent patterns raise the question of whether a general genetic mechanism often underlies the evolution of abnormal growth in mammalian hybrids. Few data have been collected to directly address whether there are general genetic trends underlying parent-of-origin growth phenotypes, but evidence from species pairs of house mice and deer mice do support the hypothesis that placental dysfunction during early development results in abnormal prenatal growth as well as adult size in hybrids (Dawson 1965). Thus placental dysfunction is an excellent candidate mechanism to explain the patterns of abnormal growth found across mammals.

Of all the tissues in a mammal, the placenta shows the highest rate of structural evolution (Leiser and Kaufmann 1994). Though despite gross morphological differences between different mammals the function of the placenta remains the same: a conduit through which offspring derive all the essential nutrients directly from the maternal tissue (Wildman 2011). Such a close interaction between two different genomes as that at the maternal-fetal interface can result in conflict over resource allocation (Burt and Trivers 2008). Conflict theory predicts that offspring strategies to garner more resources are met with maternal countermeasures to evenly allocate

them (Haig 2002; Crespi and Semeniuk 2004; D. W. Zeh and Zeh 2000). This arms race is played out in the placenta and predicts that placental-expressed genes should show rapid rates of molecular evolution (Burt and Trivers 2008; Haig 1993). Surprisingly, the growth controlling genes expressed in the placenta *Igf2* and *Igf2r* show no sign of rapid evolution in their coding regions (McVean and Hurst 1997; Smith and Hurst 1998; Smith and Hurst 1999). Though controversial, it seems that the outcome of placental conflict has instead been the origin of a unique mode of gene regulation: genomic imprinting (Moore and Haig 1991) (but see: Solter 1988; Wolf and Hager 2006; Varmuza and Mann 1994; Sapienza 1989; Hall 1990; Barlow 1993).

Genomic imprinting has recently evolved in mammals as a mode of gene regulation that involves the silencing of one allele based on its parent of origin (Surani et al. 1990). This unusual mode of gene regulation presents a possible resolution to the conflict over resource allocation between the maternal and paternal genomes (Moore and Haig 1991). Genes that tend to promote embryonic growth are often paternally expressed while genes that tend to inhibit excess embryonic growth are often maternally expressed (Morison, Ramsay, and Spencer 2005). Along with genes that influence growth, the paternally derived X chromosome is also silenced in the extraembryonic tissue in females (Harper, Fosten, and Monk 1982). This is called imprinted X chromosome inactivation (XCI). Imprinted genes are commonly involved in placental formation and are crucial for proper placental function (Piedrahita 2011).

There are two reasons that imprinted genes are likely to be involved in reproductive isolation. First, imprinted genes evolve under strong parent-offspring conflict (Haig and Trivers 1995; Burt and Trivers 2008) and thus are likely to evolve rapidly. Rapid evolution results in interspecific differences in patterns of imprinting and may increase sensitivity to regulatory



disruption (Varmuza 1993). Disruption of the regulatory silencing of a single allele results in abnormal development when the dosage balance between growth factors and repressors is skewed (Vrana 2007). Indeed, aberrant expression of imprinted genes is associated with abnormal placenta morphology and extreme growth in both deer mice (Duselis and Vrana 2007; Duselis and Vrana 2010; Vrana et al. 1998) and house mice (Shi et al. 2004; Zechner et al. 1997). However, while these two systems have quite similar growth phenotypes, they are caused by strikingly different genetic mechanisms. Hybrid dysgenesis in deer mice has been linked to an epistatic interaction between loss of imprinting at the paternally expressed gene *Peg3* and an X-linked loci, *Esx1* (Vrana et al. 2000; Loschiavo et al. 2007) and wide-scale loss of maternal imprinting is also found in overgrown offspring. In house mice the imprinting of *Peg3* is also disrupted, but a backcross mapping panel showed that the expression pattern of *Peg3* was not associated with abnormal growth (Zechner et al. 2004). As these two systems show markedly different genetic mechanisms despite having similar phenotypes, the addition of a third system is well motivated to establish common themes underlying mammalian reproductive isolation.

A second reason that imprinted genes are likely to be involved in hybrid inviability is that they are expressed from a single chromosome. Even though there are two genomic copies of each imprinted gene, they are functionally haploid. In this way imprinted genes are similar to the hemizygous X chromosome in males: they both could result in the exposure of recessive incompatibilities in hybrids that would otherwise be masked (Turelli and Orr 1995). This is one of the reasons for Haldane's rule and the same logic can be applied to any haploid-expressed gene. Indeed when small sections of autosomes were made hemizygous through deletion lines in *Drosophila*, they resulted in hybrid inviability (Coyne, Simeonidis, and Rooney 1998; Presgraves et al. 2003). Furthermore, imprinting is intriguing in light of the DMI model as

interactions occur between haploid-expressed (imprinted) alleles from different species thus uncovering recessive interactions. Indeed, many paternally expressed genes interact directly with maternally expressed genes, a good example of which is *Igf2*, a paternally expressed growth promoter and its repressor, *Igf2r*, which is maternally expressed. The imprinted nature of these loci forces alleles from opposite species to interact with each other, which increases the probability that an incompatibility may occur.

This dissertation dissects the developmental basis of reproductive isolation between two species of hamsters: *Phodopus campbelli* and *Phodopus sungorus*. These two species are very closely related, sharing a common ancestor ~1 million years ago (Neumann et al. 2006), and have only recently been elevated from subspecies status based on hybrid male sterility in laboratory crosses (Sokolov and Vasil'eva 1993; Safronova and Vasil'eva 1996; Ishishita et al. 2015). Dwarf hamsters are native to central Asia and the ranges are not reported to overlap, though sampling has been somewhat sparse. Furthermore, they are reported to show parent-of-origin dependent growth where one hybrid is much larger than the parents while the reciprocal hybrid is similar in size to the parents (Sokolov and Vasil'eva 1993; Safronova and Vasil'eva 1996). I have carried out a series of experiments to dissect the developmental basis of hybrid inviability between these two species of dwarf hamster. I first used reciprocal crosses to describe the exact pattern of developmental breakdown in hybrids. Then I assayed gene expression across the hybrid placental transcriptome to determine whether gene regulation is disrupted. Finally, I used a backcrosses mapping panel to identify the regions of the genome that are responsible for abnormal development. Thus, my dissertation uses classical genetics combined with next-generation sequencing technology to directly link genotype to phenotype.

- Abzhanov, Arhat, Meredith Protas, B Rosemary Grant, Peter R Grant, and Clifford J Tabin. 2004. “*Bmp4* And Morphological Variation of Beaks in Darwin's Finches.” *Science* 305 (5689): 1462–65. doi:10.1126/science.1098095.
- Allen, W R, J A Skidmore, F Stewart, and D F Antczak. 1993. “Effects of Fetal Genotype and Uterine Environment on Placental Development in Equids.” *Reproduction* 98 (1): 55–60. doi:10.1530/jrf.0.0980055.
- Barlow, Denise P. 1993. “Methylation and Imprinting: From Host Defense to Gene Regulation?.” *Science* 260 (5106): 309–10.
- Bateson, William. 1909. “Heredity and Variation in Modern Lights.” In *Darwin and Modern Science*, edited by A C Seward, 85–101. Cambridge, MA: Cambridge Univ Press.
- Bolker, J A. 2000. “Modularity in Development and Why It Matters to Evo-Devo.” *American Zoologist* 40 (5). Soc Integ Comp Biol: 770–76.
- Brawand, David, Magali Soumillon, Anamaria Necsulea, Philippe Julien, Gábor Csárdi, Patrick Harrigan, Manuela Weier, et al. 2011. “The Evolution of Gene Expression Levels in Mammalian Organs.” *Nature* 478 (7369):343–48. doi:10.1038/nature10532.
- Brideau, N J, H A Flores, J Wang, and S Maheshwari. 2006. “Two Dobzhansky-Muller Genes Interact to Cause Hybrid Lethality in *Drosophila*.” *Science* 314: 1292–95.
- Burt, A, and R Trivers. 2008. *Genes in Conflict: the Biology of Selfish Genetic Elements*. Boston, MA: First Harvard University Press.
- Carroll, Sean B. 2008. “Evo-Devo and an Expanding Evolutionary Synthesis: a Genetic Theory of Morphological Evolution.” *Cell* 134 (1): 25–36. doi:10.1016/j.cell.2008.06.030.
- Cohn, M J, and C Tickle. 1999. “Developmental Basis of Limblessness and Axial Patterning in Snakes.” *Nature* 399 (6735):474–78.

- Coyne, Jerry A, and H Allen Orr. 1989. "Two Rules of Speciation." In *Speciation and Its Consequences*, edited by D Otte and J A Endler, 180–207. Sunderland, MA: Sinauer Associates Inc.
- Coyne, Jerry A, and H Allen Orr. 2004. *Speciation*. Sunderland, MA: Sinauer Associates Inc.
- Coyne, Jerry A, S Simeonidis, and P Rooney. 1998. "Relative Paucity of Genes Causing Inviability in Hybrids Between *Drosophila Melanogaster* and *D. Simulans*.." *Genetics* 150 (3): 1091–1103.
- Crespi, Bernard J, and Christina Semeniuk. 2004. "Parent–Offspring Conflict in the Evolution of Vertebrate Reproductive Mode." *The American Naturalist* 163 (5): 635–53.  
doi:10.1086/382734.
- Dawson, W D. 1965. "Fertility and Size Inheritance in a *Peromyscus* Species Cross." *Evolution* 19 (1):44–55.
- Dobzhansky, T G. 1936. "Studies on Hybrid Sterility. II. Localization of Sterility Factors in *Drosophila Pseudoobscura* Hybrids." *Genetics* 21 (2):113.
- Duselis, Amanda R, and P B Vrana. 2007. "Assessment and Disease Comparisons of Hybrid Developmental Defects." *Human Molecular Genetics* 16 (7): 808–19.  
doi:10.1093/hmg/ddm025.
- Duselis, Amanda R, and Paul B Vrana. 2010. "Aberrant Growth and Pattern Formation in *Peromyscus* Hybrid Placental Development.." *Biology of Reproduction* 83 (6): 988–96.  
doi:10.1095/biolreprod.110.085654.
- Gray, Annie P. 1972. *Mammalian Hybrids*. 2nd ed. Slough, UK: Commonwealth Agricultural Bureau.

- Haig, D. 1993. "Genetic Conflicts in Human Pregnancy." *Quarterly Review of Biology* 68 (4):495–532.
- Haig, D. 2002. *Genomic Imprinting and Kinship*. Piscataway, NJ: Rutgers Univ. Press.
- Haig, David, and R Trivers. 1995. "The Evolution of Parental Imprinting: a Review of Hypotheses." In *Genomic Imprinting: Causes and Consequences*, edited by Rolf Ohlsson, K Hall, and M Ritzen, 1st ed., 17–29. Cambridge: Cambridge Univ Press.
- Haldane, J B S. 1922. "Sex Ratio and Unisexual Sterility in Hybrid Animals." *Journal of Genetics* 12 (2):101–9.
- Hall, J G. 1990. "Genomic Imprinting: Review and Relevance to Human Diseases.." *The American Journal of Human Genetics* 46:857–73.
- Harper, M I, M Fosten, and M Monk. 1982. "Preferential Paternal X Inactivation in Extraembryonic Tissues of Early Mouse Embryos." *Journal of Embryology and Experimental Morphology* 67:127–35.
- Ishishita, Satoshi, Kazuma Tsuboi, Namiko Ohishi, Kimiyuki Tsuchiya, and Yoichi Matsuda. 2015. "Abnormal Pairing of X and Y Sex Chromosomes During Meiosis I in Interspecific Hybrids of *Phodopus Campbelli* and *P. Sungorus*." *Scientific Reports* 5 (March): 9435–39. doi:10.1038/srep09435.
- Leiser, R, and P Kaufmann. 1994. "Placental Structure: in a Comparative Aspect.." *Experimental and Clinical Endocrinology* 102 (3): 122–34. doi:10.1055/s-0029-1211275.
- Lifschytz, E, and D L Lindsley. 1972. "The Role of X-Chromosome Inactivation During Spermatogenesis." *Proceedings of the National Academy of Sciences* 69 (1): 182–86.

- Loschiavo, Maria, Quang K Nguyen, Amanda R Duselis, and Paul B Vrana. 2007. "Mapping and Identification of Candidate Loci Responsible for *Peromyscus* Hybrid Overgrowth." *Mammalian Genome* 18 (1): 75–85. doi:10.1007/s00335-006-0083-x.
- Mallarino, R, O Campàs, J A Fritz, Kevin J Burns, Olivia G Weeks, Michael P Brenner, and Arhat Abzhanov. 2012. "Closely Related Bird Species Demonstrate Flexibility Between Beak Morphology and Underlying Developmental Programs." *Proceedings of the National Academy of Sciences* 109 (40): 16222–27. doi:10.1073/pnas.1206205109/-/DCSupplemental.
- McVean, G T, and Laurence D Hurst. 1997. "Molecular Evolution of Imprinted Genes: No Evidence for Antagonistic Coevolution.." *Proceedings of the Royal Society B: Biological Sciences* 264 (1382): 739–46. doi:10.1098/rspb.1997.0105.
- Mihola, O, Z Trachtulec, C Vlcek, J C Schimenti, and J Forejt. 2009. "A Mouse Speciation Gene Encodes a Meiotic Histone H3 Methyltransferase." *Science* 323 (5912): 373–75. doi:10.1126/science.1163601.
- Moore, Tom, and D Haig. 1991. "Genomic Imprinting in Mammalian Development: a Parental Tug-of-War.." *Trends in Genetics* 7 (2): 45–49. doi:10.1016/0168-9525(91)90230-N.
- Morison, Ian M, Joshua P Ramsay, and Hamish G Spencer. 2005. "A Census of Mammalian Imprinting." *Trends in Genetics* 21 (8): 457–65. doi:10.1016/j.tig.2005.06.008.
- Muller, H J. 1942. "Isolating Mechanisms, Evolution, and Temperature." *Biological Symposia* 6: 71–125.
- Neumann, Karsten, Johan Michaux, Vladimir Lebedev, Nuri Yigit, Ercument Colak, Natalia Ivanova, Andrey Poltoraus, et al. 2006. "Molecular Phylogeny of the Cricetinae Subfamily Based on the Mitochondrial Cytochrome *B* And 12S rRNA Genes and the

- Nuclear vWF Gene.” *Molecular Phylogenetics and Evolution* 39 (1): 135–48.  
doi:10.1016/j.ympev.2006.01.010.
- Orr, H Allen. 1987. “Genetics of Male and Female Sterility in Hybrids of *Drosophila Pseudoobscura* and *D. Persimilis*.” *Genetics* 116: 555–63.
- Orr, H Allen. 1996. “Dobzhansky, Bateson, and the Genetics of Speciation..” *Genetics* 144 (4): 1331–35.
- Orr, H Allen, L D Madden, Jerry A Coyne, R Goodwin, and R S Hawley. 1997. “The Developmental Genetics of Hybrid Inviability: a Mitotic Defect in *Drosophila* Hybrids..” *Genetics* 145 (4): 1031–40.
- Phadnis, N, and H Allen Orr. 2009. “A Single Gene Causes Both Male Sterility and Segregation Distortion in *Drosophila* Hybrids.” *Science* 323 (5912):376–79.
- Piedrahita, Jorge A. 2011. “The Role of Imprinted Genes in Fetal Growth Abnormalities.” Edited by Robert M Greene and M Michele Pisano. *Birth Defects Research a: Clinical Molecular Teratology* 91 (8): 682–92. doi:10.1002/bdra.20795.
- Presgraves, D C, L Balagopalan, S M Abmayr, and H Allen Orr. 2003. “Adaptive Evolution Drives Divergence of a Hybrid Inviability Gene Between Two Species of *Drosophila*.” *Nature* 423 (6941): 715–19.
- Presgraves, Daven C. 2010. “The Molecular Evolutionary Basis of Species Formation..” *Nature Reviews Genetics* 11 (3): 175–80. doi:10.1038/nrg2718.
- Safronova, L D, and N Y Vasil'eva. 1996. “Meiotic Abnormalities in Interspecific Hybrids Between *Phodopus Sungorus* (Pallas, 1773) and *Ph. Campbelli* (Thomas, 1905).” *Russian Journal of Genetics* 32 (4):486–94.

- Sapienza, Carmen. 1989. "Genome Imprinting and Dominance Modification." *Annals of the New York Academy of Sciences* 564 (1 Regulation of): 24–38. doi:10.1111/j.1749-6632.1989.tb25885.x.
- Sawamura, K, and M T Yamamoto. 1993. "Cytogenetical Localization of *Zygotic Hybrid Rescue (Zhr)*, A *Drosophila Melanogaster* Gene That Rescues Interspecific Hybrids From Embryonic Lethality.." *Molecular & General Genetics : MGG* 239 (3): 441–49.
- Shapiro, M D, M E Marks, C L Peichel, B K Blackman, K S Nereng, B Jónsson, D Schluter, and D M Kingsley. 2004. "Genetic and Developmental Basis of Evolutionary Pelvic Reduction in Threespine Sticklebacks." *Nature* 428 (6984):717–23.
- Shi, Wei, Louis Lefebvre, Yang Yu, Sabine Otto, Antje Krella, Annie Orth, and Reinald H Fundele. 2004. "Loss-of-Imprinting of *Pegl* In Mouse Interspecies Hybrids Is Correlated with Altered Growth." *Genesis* 39 (1): 65–72. doi:10.1002/gene.20027.
- Smith, N G C, and Laurence D Hurst. 1998. "Molecular Evolution of an Imprinted Gene: Repeatability of Patterns of Evolution Within the Mammalian Insulin-Like Growth Factor Type II Receptor." *Genetics* 150 (2)823–33.
- Smith, N G, and Laurence D Hurst. 1999. "The Causes of Synonymous Rate Variation in the Rodent Genome. Can Substitution Rates Be Used to Estimate the Sex Bias in Mutation Rate?." *Genetics* 152 (2): 661–73.
- Sokolov, V E, and N I Vasil'eva. 1993. "Hybridologic Analysis Confirms the Species Specificity of *Phodopus Sungorus* (Pallus, 1773) and *Phodopus Campbelli* (Thomas, 1905)." *Doklady Akademii Nauk* 332 (1): 120–23.
- Solter, D. 1988. "Differential Imprinting and Expression of Maternal and Paternal Genomes.." *Annual Review of Genetics* 22 (1): 127–46. doi:10.1146/annurev.ge.22.120188.001015.



- Surani, M A, R Kothary, N D Allen, and P B Singh. 1990. "Genome Imprinting and Development in the Mouse." *Development Supplement*, 89–98.
- Turelli, M, and H Allen Orr. 1995. "The Dominance Theory of Haldane's Rule.." *Genetics* 140 (1): 389–402.
- Varmuza, S. 1993. "Gametic Imprinting as a Speciation Mechanism in Mammals.." *Journal of Theoretical Biology* 164 (1): 1–13. doi:10.1006/jtbi.1993.1137.
- Varmuza, S, and M Mann. 1994. "Genomic Imprinting-Defusing the Ovarian Time Bomb.." *Trends in Genetics* 10 (4): 118–23.
- Vrana, P B. 2007. "Genomic Imprinting as a Mechanism of Reproductive Isolation in Mammals." *Journal of Mammalogy* 88 (1): 5–23.
- Vrana, P B, J A Fossella, P Matteson, T del Rio, M J O'Neill, and S M Tilghman. 2000. "Genetic and Epigenetic Incompatibilities Underlie Hybrid Dysgenesis in *Peromyscus*.." *Nature Genetics* 25 (1): 120–24. doi:10.1038/75518.
- Vrana, P B, X J Guan, R S Ingram, and S M Tilghman. 1998. "Genomic Imprinting Is Disrupted in Interspecific *Peromyscus* Hybrids.." *Nature Genetics* 20 (4): 362–65. doi:10.1038/3833.
- White, M A, B Steffy, T Wiltshire, and B A Payseur. 2011. "Genetic Dissection of a Key Reproductive Barrier Between Nascent Species of House Mice." *Genetics* 189 (1): 289–304. doi:10.1534/genetics.111.129171.
- White, M A, M Stubbings, B L Dumont, and B A Payseur. 2012. "Genetics and Evolution of Hybrid Male Sterility in House Mice." *Genetics* 191 (3): 917–34. doi:10.1534/genetics.112.140251.

- Wildman, D E. 2011. "Review: Toward an Integrated Evolutionary Understanding of the Mammalian Placenta." *Placenta* 32 (S2):S142–45. doi:10.1016/j.placenta.2011.01.005.
- Wolf, Jason B, and R Hager. 2006. "A Maternal–Offspring Coadaptation Theory for the Evolution of Genomic Imprinting." *PLoS Biology*. doi:10.1371/journal.pbio.0040380.st001.
- Zechner, U, Matthais Reule, P S Burgoyne, A Schubert, Annie Orth, H Hameister, and Reinald H Fundele. 1997. "Paternal Transmission of X-Linked Placental Dysplasia in Mouse Interspecific Hybrids." *Genetics* 146 (4):1399–1405.
- Zechner, U, W Shi, M Hemberger, H Himmelbauer, Sabine Otto, Annie Orth, V Kalscheuer, et al. 2004. "Divergent Genetic and Epigenetic Post-Zygotic Isolation Mechanisms in *Mus* and *Peromyscus*." *Journal of Evolutionary Biology* 17 (2): 453–60. doi:10.1046/j.1420-9101.2003.00656.x.
- Zeh, D W, and J A Zeh. 2000. "Reproductive Mode and Speciation: the Viviparity-Driven Conflict Hypothesis." *BioEssays* 22 (10): 938–46. doi:10.1002/1521-1878(200010)22:10<938::AID-BIES9>3.0.CO;2-9.

**PARENT-OF-ORIGIN GROWTH EFFECTS AND THE EVOLUTION OF HYBRID  
INVIABILITY IN DWARF HAMSTERS**

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## Abstract

Mammalian hybrids often show abnormal growth, indicating that developmental inviability may play an important role in mammalian speciation. Yet it is unclear if this recurrent phenotype reflects a common genetic basis. Here we describe patterns of hybrid inviability between two closely related species of dwarf hamsters, *Phodopus campbelli* and *P. sungorus*. Using genetic crosses, we found extreme parent-of-origin dependent growth in hybrid embryos and placentas. One cross type resulted in massive overgrowth, severe developmental defects, and prenatal and maternal death. Embryos from the reciprocal cross were viable and normal in size but adult hybrid males were smaller than either species. These effects are strikingly similar to patterns reported from other mammalian hybrids and demonstrate that extreme hybrid growth can evolve rapidly. Next we tested the hypothesis that parent-of-origin growth effects in hybrids result from the disruption of genomic imprinting. We found no association between patterns of expression at several candidate imprinted genes and parent-of-origin growth effects, thus excluding widespread loss of imprinting in hybrids. However, our data do not rule out loss of imprinting at other genes. Collectively, our study indicates that growth-related hybrid inviability may be common in mammals but that the genetic underpinnings of these phenotypes remain unresolved.

## Introduction

Considerable progress has been made on understanding the evolution of genetic interactions that lead to reduced fertility or viability of hybrids (i.e., intrinsic postzygotic reproductive isolation). These efforts have yielded several fundamental insights into the evolution of intrinsic reproductive isolation (reviewed in Coyne and Orr 2004), including that hybrid incompatibilities often result from deleterious interactions between divergent genes (Dobzhansky 1937; Muller 1942; Orr 1996; Brideau et al. 2006; Tang and Presgraves 2009) and that epistatic interactions involving the sex chromosomes evolve very rapidly (Coyne and Orr 1989b; Masly and Presgraves 2007). Some progress has also been made in linking these general genetic patterns underlying hybrid male sterility to the disruption of specific developmental processes during spermatogenesis (Good et al. 2010; Meiklejohn et al. 2011; Campbell et al. 2013; Bhattacharyya et al. 2013). However, much less headway has been made on the evolution of inviability and we remain relatively ignorant to the simple question of if specific developmental pathways are predisposed to disruption in animal hybrids (Coyne and Orr 2004).

It is unclear whether we should even expect a single pathway to play a recurrent role in hybrid inviability as processes acting early during embryogenesis tend to be widely conserved between species (Coyne and Orr 2004). Consistent with this, hybrid lethality tends to evolve more slowly than hybrid sterility in some species (Coyne and Orr 1989a; 1997), leading some to question the relevance of inviability to the early stages of speciation (Sobel et al. 2010). Nonetheless, regulatory changes influencing diverse aspects of morphological development can evolve very rapidly (e.g., Cohn and Tickle 1999; Abzhanov et al. 2004; Shapiro et al. 2004; Mallarino et al. 2012), and thus could also play an

important role in the evolution of reproductive isolation between closely related species. In particular, the mammalian radiation is a compelling system in which to study the evolution of development in the context of speciation. Mammals show great morphological diversity between species and hybrid inviability arises at a comparatively rapid rate (Prager and Wilson 1975; Fitzpatrick 2004). Furthermore, some mammal hybrids show abnormal patterns of growth (Table 1) and reduced fitness (Gray 1972). Interestingly, some of these hybrids show parent-of-origin dependent growth; one hybrid is larger than the parents while the reciprocal hybrid is equal to or smaller than the parents (Dawson 1965; Allen 1969; Rogers and Dawson 1970; Allen et al. 1993; Zechner et al. 1996). These recurrent phenotypes raise the questions of whether disruption of a general developmental process often underlies the evolution of hybrid inviability in mammals and if these intrinsic incompatibilities evolve rapidly enough to play an important role in mammalian speciation.

Most of the evidence for abnormal growth in mammals derives from casual descriptions of captive hybrids that showed extreme adult sizes relative to their parent species (Gray 1972). However, a few in depth studies have shown that hybrid growth effects are associated with abnormal placentation during mid-gestation (Dawson 1965; Rogers and Dawson 1970; Zechner et al. 1996; Vrana et al. 1998). The placenta is derived largely from embryonic tissue and acts as a conduit for the transfer of maternal nutrients to the embryo. Both growth factors and their antagonists (i.e., growth repressors) are expressed in the placenta. The dosage-dependent interaction between these two classes of genes influences nutrient allocation and ultimately regulate growth in developing embryos (Haig 1996; Reik et al. 2003; Saukkonen 2004). Moreover, many placental expressed genes are controlled by an unusual mode of regulation called genomic imprinting, which results

in the epigenetic silencing of one allele depending on its parent of origin (Surani et al. 1990). Imprinted genes are commonly involved in placental formation (Piedrahita 2011) and show a functional bias where paternally expressed (i.e., maternally imprinted) genes tend to promote embryonic growth while maternally expressed (i.e., paternally imprinted) genes repress growth (Morison et al. 2005). Thus, abnormal hybrid growth in mammals may generally reflect disrupted placental function caused by a failure of imprinted genes to properly interact with each other in the hybrid placenta (Vrana 2007).

There are three key reasons why imprinted placental genes are intriguing in the context of speciation. First, the placenta is likely subject to intense evolutionary conflict over resource allocation (Burt and Trivers 1998) because it mediates interactions between two different genomes (maternal and paternal/offspring). Conflict is particularly relevant in the case of multiple paternity (Haig 1999) where offspring strategies should evolve to garner more resources at the expense of their half-siblings and maternal countermeasures are expected to assure even allocation to all offspring (Zeh and Zeh 2000; Haig 2002; Crespi and Semeniuk 2004). Consistent with these predictions, the placenta shows the highest rate of structural evolution of all mammalian tissues (Leiser and Kaufmann 1994). Second, loss of imprinting at a single gene can skew the dosage balance between growth factors and repressors, causing abnormal development and pronounced growth (Li et al. 1999). In turn, rapid divergence of imprinting patterns between species is predicted to cause dosage imbalance in hybrids (Varmuza 1993). Third, imprinted genes are expressed from a single chromosome and are thus functionally haploid. Similar to the well-known differential exposure of recessive X-linked hybrid incompatibilities in males (Muller 1942; Turelli and Orr 1995; 2000), haploid expression could expose recessive incompatibilities

that would otherwise be masked in hybrids. Furthermore, epistatic interactions between recessive hybrid incompatibilities would also be differentially exposed in F1 hybrids because many paternally and maternally imprinted genes directly interact (Czech 1989; Haig and Graham 1991).

Patterns of placental gene expression have been evaluated in three systems that show abnormal hybrid growth. Disrupted gene expression is associated with parent-of-origin effects on morphology and size of the placenta in both deer mice (Vrana et al. 1998; Duselis and Vrana 2007; 2010) and house mice (Zechner et al. 1996; 1997; Shi et al. 2004; Brown et al. 2012). These two systems show quite similar placental phenotypes that appear to have different genetic bases (Zechner et al. 2004). Placental dysgenesis in deer mice is caused by an epistatic interaction between loss of imprinting in at least one paternally expressed gene and the maternally expressed X chromosome (Vrana et al. 2000; Loschiavo et al. 2007), though widespread loss of maternal imprinting is also apparent in overgrown offspring (Vrana et al. 2000). Reciprocal growth effects in the placenta of hybrid house mice also are caused by an X-autosome interaction (Zechner et al. 1996; Hemberger et al. 1999) and there is some evidence for disrupted imprinting (Shi et al. 2004; 2005). However, these regulatory effects appear to be less pronounced and genetically distinct from those described in deer mice (Zechner et al. 2004). A third hybrid cross between horses and donkeys results in parent-of-origin effects for abnormal placental morphology (Allen 1969; Allen et al. 1993), but with no evidence for disrupted genomic imprinting (Wang et al. 2013). As these three systems show similar phenotypes that are caused by different genetic mechanisms, it remains to be seen how often disrupted imprinting



underlies the evolution of mammalian reproductive isolation in general and parent-of-origin growth effects in particular.

Here we describe patterns of reproductive isolation between two species of dwarf hamsters, *Phodopus sungorus* and *P. campbelli*. Dwarf hamsters are native to the xeric habitats of central Asia with *P. sungorus* occurring on the Kazakh steppe (Ross 1998) and *P. campbelli* occurring in the semi-deserts of Mongolia, northern China, and southern Russia (Ross 1995). They are sister species (Neumann et al. 2006) that have only recently been elevated from subspecies status based primarily on evidence of sterility in hybrid males (Sokolov and Vasil'eva 1993; Safronova and Vasil'eva 1996; Safronova et al. 1999). In addition, one direction of the cross (female *P. sungorus* × male *P. campbelli*) has been reported to result in “heterotic” hybrids with exaggerated growth and an increased incidence of unspecified birth defects (Safronova and Vasil'eva 1996). These observations suggest that dwarf hamsters may provide a novel system with which to evaluate the developmental basis of abnormal hybrid growth between mammal species still in the early stages of divergence. We have two primary objectives. First, we use reciprocal crosses to test for hybrid inviability phenotypes, with a specific focus on parent-of-origin growth effects throughout the lifecycle of F<sub>1</sub> hybrids. Second, we examine patterns of expression at eight candidate genes to test for disrupted genomic imprinting in hybrid placenta. We discuss our findings within the broader context of the developmental mode and evolutionary tempo of growth-related hybrid inviability in mammals.

## Methods

### *Animals*

Outbred dwarf hamster colonies were established at the University of Montana in the fall of 2011 using six mating pairs of *P. campbelli* provided by Robert Johnston and six mating pairs of *Phodopus sungorus* provided by Ned Place, both from Cornell University. Both of these stocks were derived from natural populations sampled by Catherine Wynne-Edwards in 1981 and most recently supplemented with additional wild hamsters in 1990 (Scribner and Wynne-Edwards 1994). We have maintained our breeding colonies using a crossing scheme designed to minimize inbreeding (Wright 1921) and all crosses used in this experiment were within the first five generations of our colony. All animals were housed in 14L:10D light/dark regimen and in accordance with IACUC regulations.

### *Experimental crosses and phenotypic analyses*

We conducted a total of 331 experimental crosses within and between the two species: 1) 110 *P. campbelli* × *P. campbelli*, 2) 88 *P. campbelli* × *P. sungorus*, 3) 32 *P. sungorus* × *P. campbelli*, and 4) 101 *P. sungorus* × *P. sungorus*, where the female is always specified first. These crosses were used to collect a suite of developmental phenotypes described below.

First, we collected late-term embryos and placentas from euthanized pregnant females to determine the frequency and extent of developmental defects. Dwarf hamsters have an 18-day gestation period with a facultative delay of up to four days due to developmental diapause and/or delayed implantation (Newkirk et al. 1997). To control for this variation, dissected embryos were developmentally staged according to a suite of established characters in golden hamsters (Boyer 1953) and mice (Butler and Juurlink

1987). For our analysis, we only used late-term embryos corresponding roughly to Theiler's Stages 24-27 of mouse development (Theiler 1972). Litter size was recorded and dissected embryos and placentas were photographed, weighed and given a presence/absence score for several developmental defects including the occurrence of molar conceptuses (hydatiform moles), embryo reabsorption, embryo swelling (edema). All embryos and placenta were then snap-frozen on dry ice to preserve RNA for gene expression analyses.

Second, to determine if growth phenotypes identified *in utero* persisted throughout the animal's life cycle and to test for the emergence of new phenotypes in adults we allowed several crosses to proceed to term. To quantify mating isolation, we tested for differences in the number of successful crosses and latency to birth for adult females paired with a hetero- or conspecific male for up to 40 days. To quantify postnatal growth, we generated a standard growth curve for each cross-type by weighing each offspring every ten days after birth until day 100. We modeled growth with an asymptotic curve and tested for differences in the asymptote (final adult size) between each of the cross types. *P. sungorus* × *P. campbelli* hybrids could not be brought to term and were excluded from these experiments (see below).

Phenotypic data has been deposited in Dryad and all statistical analyses were performed using R version 3.0.2 (R Core Team 2008). We calculated both one-way analyses of variance (ANOVA) and non-parametric pairwise Wilcoxon rank-sum tests for all comparisons between cross types. Results of the Wilcoxon test are reported for phenotypes (e.g., embryo and placental weights) with large differences in variance between the groups.

Multiple comparisons were accounted for by using a Bonferroni correction when appropriate.

#### *Genetic sex-typing of embryos*

The sex of hybrid embryos was determined by Polymerase Chain Reaction (PCR) amplification and Sanger sequencing of a 764 bp fragment of the X-linked gene *Zfx*. Degenerate primers were designed for dwarf hamsters by modifying the generic LGL331 and LGL335 primers of Shaw and colleagues (2003) based on an alignment of *Zfx* and *Zfy* sequences from rat, house mouse, guinea pig, Golden hamster, and Chinese hamster (see Supplemental Table 2 for accession numbers). The *Zfx/Zfy* sex-typing system usually relies upon a diagnostic intron length polymorphism between homologous genes on the X (*Zfx*) and Y (*Zfy*) (Shaw et al. 2003), but we were unable to amplify *Zfy* in *Phodopus*. Therefore, we sequenced *Zfx* and identified five fixed nucleotide differences between the species that we then assayed in hybrids. Heterozygous hybrids were classified as female and homozygous hybrids possessing the expected maternal genotype were classified as male. We verified the accuracy of our assay by typing several adult hybrids of known sex; however, the sex of non-hybrid individuals could not be determined using this approach. All primer sequences and PCR reaction conditions used in this study can be found in Supplemental Table 2. Sequence alignments were performed using the program Geneious (version 6.1.5; Drummond et al. 2005).

#### *Genetic divergence between hybridizing mammal species*

We compiled mitochondrial cytochrome *b* (cyt *b*) sequence data for 36 species pairs that have been reported to show some degree of abnormal hybrid growth (Supplemental Table 1). For dwarf hamsters, we designed primers to amplify and sequence 910 base pairs of cyt *b* in both species (Supplemental Table 2). For the other 35 species pairs we used previously published cyt *b* sequences from GenBank (see Supplemental Table 1 for accession numbers); five species pairs did not have available data for cyt *b*. After trimming positions with missing data, 34 species pairs shared a common 718 base pair alignment that we used to calculate Kimura two-parameter-corrected pairwise divergences (Supplemental Table 1) using the program Phylip (version 3.6a3, Felsenstein 2002).

#### *Qualitative survey of gene expression*

We targeted eight genes that show imprinted expression in the placenta of house mice (Morison et al. 2005), including four paternally expressed genes (maternally imprinted *Igf2*, *Mest*, *Peg3*, *Snrpn*) and four maternally expressed genes (paternally imprinted *H19*, *Igf2r*, *Grb10*, and *Mash2*). These candidates were selected because several of them show disrupted placental imprinting in hybrid deer mice (Vrana et al. 1998). Primers were designed using Primer3 (Rozen and Skaletsky 2000) based on exon sequences aligned between mouse, human, rat, and guinea pig (Supplemental Table 2). Amplicons were designed to span at least one intron in five of the genes to minimize the risk of genomic DNA contamination. PCR products for *Peg3*, *H19* and *Mash2* did not span introns because either no conserved priming sites could be found or no diagnostic site was present in the amplicon.

We assayed expression of these genes by sequencing complementary DNA (cDNA) from 24 late-gestation placentas, including three from each species and six (three male, three female) from each reciprocal hybrid. Whole placentas were homogenized in liquid nitrogen with a mortar and pestle and total RNA was extracted using an E.Z.N.A. Total RNA Kit (Omega) treated with DNase, and converted to cDNA with the cDNA Supermix Kit (Quantas). Exonic regions were then PCR amplified from cDNA, Sanger sequenced, and examined for fixed differences between the species. All eight loci are autosomal in house mice, therefore, hybrid individuals should be heterozygous at all diagnostic positions in the absence of imprinting. Using this rationale, we classified gene expression in hybrids as imprinted (homozygous for the maternal or paternal allele) or biallelic (heterozygous). As with the sex-typing assay, this assay is only effective in the F<sub>1</sub> hybrids. Imprinted expression was called only when a single peak from the expected allele was visible on the chromatogram (Supplemental Figure 1). This is a conservative metric given that imprinting sometimes results in skewed biallelic expression (Babak et al. 2008) .

All PCR products were Sanger sequenced at the University of Montana Murdock Lab DNA Sequencing Facility or the University of Arizona Genetics Core and have been deposited in GenBank under the accession numbers JX217832-JX217849, JX436485-JX436486, and KF673394.1-KF673395.1.

## Results

### *Reduced pregnancy rate in hybrid crosses*

Pregnancy rates were similar and relatively high within each species. Females became pregnant in 83% (67 of 81) of *P. campbelli* crosses and 84% (68 of 81) of *P. sungorus* crosses. In contrast, *P. campbelli* females were successfully impregnated by a *P. sungorus* male only 68% of the time (43 of 63 crosses;  $P=0.017$ , Fisher's Exact Test (FET) versus pooled species, Bonferroni corrected  $\alpha=0.025$ ). The pregnancy rate was also marginally reduced in the reciprocal cross (*P. sungorus*  $\times$  *P. campbelli*; 66% or 19 of 29 crosses;  $P=0.038$  FET versus pooled species). When considering the subset of pairs that reached parturition, we found no reduction in the average latency from pairing to birth for successful heterospecific pregnancies relative to conspecific matings. *Phodopus campbelli* and *P. sungorus* averaged 24.2 days (62 crosses) and 23.3 (63 crosses) days respectively from pairing to birth, while the hybrid cross *P. campbelli*  $\times$  *P. sungorus* averaged 22.2 days (37 crosses). The reciprocal hybrid cross, *P. sungorus*  $\times$  *P. campbelli*, did not yield any successful births (see below).

### *Parent-of-origin effects with extreme asymmetric hybrid overgrowth*

We found that the mean weight of hybrid embryos from a *P. sungorus* mother and a *P. campbelli* father was ~38% larger than any other cross (Figure 1A,  $P<0.001$ , Wilcoxon rank-sum test, Bonferroni corrected  $\alpha=0.008$ ). Parent-of-origin dependent growth was even more striking in the placenta (Figure 1B, Supplemental Figure 2); placentas derived from a female *P. sungorus* and a male *P. campbelli* father were around 300% heavier than placentas from any other cross-type (Figure 1B,  $P<0.001$ , Wilcoxon rank-sum test,

Bonferroni corrected  $\alpha=0.008$ ). We found no sex-specific differences in the placenta or embryo weights of either reciprocal hybrid type (*P. sungorus*  $\times$  *P. campbelli*: placenta  $P=0.175$ , embryo  $P=0.109$ ; *P. campbelli*  $\times$  *P. sungorus*: placenta  $P=0.880$ , embryo  $P=0.880$ ; Wilcoxon rank-sum tests, Bonferroni corrected  $\alpha=0.013$ ). Following Vrana and colleagues (1998) we will hereafter refer to the large *P. sungorus*  $\times$  *P. campbelli* hybrids as “SXC” and the reciprocal *P. campbelli*  $\times$  *P. sungorus* hybrids as “cXs” where the species are designated by the first letter of their specific epithet, the maternal species is listed first, and the capitalization reflects the relative size of the hybrid offspring.

The overgrown SXC offspring were also more often affected by severe developmental defects. We found an elevated proportion of molar conceptuses and reabsorbing embryos in SXC crosses relative to all other cross types (Table 2). Also known as hydatiform moles, molar conceptuses are a form of placental pathology characterized by excessive extra-embryonic (placenta) tissue and no embryonic tissue (Supplemental Figure 2D: Lindor et al. 1992). Twenty-five percent of SXC embryos (18 of 73) showed edema, characterized by mild to extreme swelling (Supplemental Figure 2C), whereas embryonic edema was comparably rare in all other crosses (Table 2). Thus, abnormal *in utero* development was largely restricted to extreme overgrowth in SXC hybrids, with ~70% (53 out of 73) of SXC embryos afflicted by severe developmental defects (even when excluding embryos with edema, SXC offspring were significantly overgrown). In contrast, cXs hybrids were not significantly different than *P. campbelli* and *P. sungorus* for any of the *in utero* developmental phenotypes that we considered. However, late-gestation hybrid litters were smaller than intraspecific litters (Table 2).



The lack of significant undergrowth phenotypes in late-term cXs hybrids (Figure 1) contrasts with described parent-of-origin effects in hybrid deer mice (Vrana et al. 1998) and house mice (Zechner et al. 1996), where significant but opposite growth effects are manifest *in utero* in reciprocal hybrid embryos and/or placentas. Our study lacks power to detect subtle weight differences at late gestation (e.g., we have only ~12% power to detect an effect size of 0.2). Because parent-of-origin effects often persist or even become exaggerated into adulthood, we next compared the postnatal growth curves of both species to the cXs hybrids (Figure 2). This experiment was initiated with 26 individuals from 6 crosses for *P. campbelli*, 38 individuals from 6 crosses for *P. sungorus*, and 45 individuals from 15 crosses for *P. campbelli* × *P. sungorus* hybrids. Though approximately the same size at late gestation (Figure 1A), adult cXs hybrids were much smaller than either parent species (Figure 2;  $F_{2,14}=77.116$ ,  $P<0.001$ ). Specifically, we found a significant reduction in adult weights of cXs males versus males of either species (Figure 3;  $P<0.001$ , *t*-test, Bonferroni corrected  $\alpha=0.017$ ). As is typical for many mammals, both species of dwarf hamsters are sexually dimorphic as adults (80 days) with males at least 10% larger than the females (Figure 3). In contrast, cXs hybrid females were larger than hybrid males (Figure 3); 80-day old cXs females were approximately the same size as their *P. campbelli* mothers whereas the males were only half as large as their fathers (Figure 3). Thus, reduced adult growth of cXs hybrids (Figure 2) appears to be driven mostly by a male-specific reduction in body weight. We did not measure postnatal growth of SXC hybrids because three attempts to birth the overgrown hybrids failed and resulted in maternal death.

Reduced postnatal growth only in cXs hybrid males is consistent with Haldane's rule, which states that inviability should affect males more often than females in male-heterogametic taxa (Haldane 1922). To further test for sex-specific inviability we analyzed the sex ratios of late-term and adult hybrids. We found no significant bias in the sex ratio of either hybrid type *in utero* (Table 2; cXs  $X^2=1.125$ ,  $df=1$ ,  $P=0.288$ ; SXC  $X^2=0.118$ ,  $df=1$ ,  $P=0.537$ , chi-squared test), but we did find significantly male-biased adult sex ratios in cXs hybrids (Table 3; 61.8% male,  $X^2=8.463$ ,  $df=1$ ,  $P<0.001$ , chi-squared test). Further inspection of the average counts for each sex suggests that this male-biased skew primarily reflects a reduction in the number of females per litter (Table 3). Finally, we tested whether one sex is differentially susceptible to molar conceptuses, reabsorption, and/or edema in the SXC hybrids. We found approximately equal numbers of males and females affected by each of these phenotypes (molar conceptuses:  $P=0.715$ ; reabsorbing embryos:  $P=1.0$ ; edema:  $P=0.169$ ,  $n=25$  females and 30 males, FET). Thus, we find a surprising pattern in adult cXs hybrids where males are more common but significantly smaller than females.

#### *No global disruption of imprinting associated with parent-of-origin growth effects*

Species-specific disruption of placental imprinting has been put forth as a general explanation for parent-of-origin dependent growth effects in reciprocal hybrids (Vrana et al. 1998; Vrana 2007). Specifically, this model predicts that (i) hybrid overgrowth results from maternal expression of one or more growth factors that are normally silenced through imprinting and (ii) that undergrowth results in the reciprocal cross when growth repressors are expressed from the normally silenced paternal genes. For a given gene, this simple model predicts that disrupted imprinting will result in biallelic expression in one

hybrid while expression in the reciprocal hybrid remains properly imprinted. Extreme overgrowth occurs in our dwarf hamster crosses when *P. sungorus* is the mother and undergrowth is manifest when *P. sungorus* is the father (Figures 1 and 2). Thus, if disrupted imprinting is the cause of these parent-of-origin effects then we should see the gain of expression of *P. sungorus* (maternal) growth factor alleles in S×C placentas, *P. sungorus* (paternal) growth repressors in c×s placentas, and proper imprinting of *P. campbelli* alleles in both hybrid crosses.

To test this general model, we assayed the expression of eight genes that are imprinted in mice and have been shown to influence embryonic growth. For each gene, we tested six placentas (three per sex) from each of the reciprocal hybrid crosses for imprinted (monoallelic) expression of the maternal or paternal allele (Supplemental Figure 1). Seven of the eight candidate genes were found to contain one or more fixed differences between the species. The paternally expressed gene *Mest* showed no fixed differences and therefore could not be assayed for allele-specific expression in hybrids. Two maternally imprinted candidates (*Igf2*, and *Snrpn*) showed expression of only the paternal allele, consistent with imprinted expression patterns in *Mus* (Table 4). *Peg3* was maternally imprinted in c×s hybrids, but showed variation in imprinting status among in S×C hybrids. Of the six S×C hybrids, one male and one female showed biallelic expression while the other four offspring exhibited imprinted expression. These results were verified in two independent cDNA preparations that showed no evidence of genomic DNA contamination. Three of the four maternally expressed genes (*Grb10*, *Igf2r*, and *Mash2*) showed biallelic expression in both reciprocal hybrids, while *H19* showed only maternal expression

consistent with paternal imprinting (Table 4, Supplemental Figure 1; Morison et al. 2005).

Patterns of expression were identical between males and females for all genes.

## Discussion

### *Parent-of-origin growth effects in dwarf hamsters and other mammals*

We have shown that dwarf hamster hybrids display strong parent-of-origin growth effects that manifest a wide range of inviability phenotypes. When a *P. sungorus* female was crossed with a *P. campbelli* male, embryo and placenta overgrowth was so extreme that it is ultimately lethal to the mother and offspring during birth. However, despite the potentially high rates of maternal mortality suggested by our study, viable SXC hybrids have been reported (Sokolov and Vasil'eva 1993). In this previous study, the average adult weight of 13 SXC hybrids was 55.2 grams — nearly 200% the weight of either species. It is unclear what the probability of a successful SXC birth is based on these limited data, but they do demonstrate that the striking placental and embryonic overgrowth that we observed during late gestation (Figure 1) persist through to adulthood. Alternatively, adult cXs hybrid males were approximately 40% smaller than their male parents though they were not significantly different in size at birth. Thus, the overgrown SXC cross yields more severe hybrid inviability phenotypes but both crosses show evidence for growth effects and reproductive isolation as evidenced by significantly reduced litter sizes (Table 2).

Parent-of-origin growth effects in hybrid dwarf hamsters are strikingly similar to several previously described examples in other hybrid mammals (Vrana 2007). Extreme and often lethal hybrid overgrowth also occurs in crosses between female *Peromyscus polionotus* and male *P. maniculatus* (Dawson 1965; Rogers and Dawson 1970; Dawson et al. 1993; Vrana 2007). Likewise, the well-known example of reciprocal crosses between lions (*Panthera leo*) and tigers (*P. tigris*) results in strong parent-of-origin growth phenotypes that persist into adulthood. So-called ligers (hybrids from a female tiger × male lion) are

reported to reach adult weights approaching 150% the size of a tiger (the larger of the parents). Gray (1972) quotes a report of one liger that “weighed as much as both parents together”, which is striking if not strongly quantitative. The reciprocal cross (i.e., tigons) is also reported to be smaller than either species (Vrana 2007). No data are available for liger or tigon placentas but we predict that their growth would be similarly affected.

Interestingly, not all hybrid crosses presenting abnormal placental growth also manifest embryonic or adult growth phenotypes. For example, parent-of-origin developmental effects in several mouse crosses (genus *Mus*) are restricted to the placenta, and do not strongly influence embryonic or adult growth (Zechner et al. 1996; Kurz et al. 1999; Zechner et al. 2004). Reciprocal crosses between horses and donkeys also yield parent-of-origin effects on placental size and morphology but not embryo size (Allen 1969; Allen et al. 1993). In both of these systems abnormal placentation impacts embryonic viability in hybrids (West et al. 1977; Zechner et al. 1996; Kurz et al. 1999; Allen 2001). Artificial insemination has recently been used to achieve a more divergent *Mus* cross that results in extreme placental and embryonic growth (*M. musculus* and *M. caroli*; Brown et al. 2012). Thus abnormal placentation appears to represent an important but not sufficient first step in the evolution of parent-of-origin growth effects in adult hybrid mammals. Most of the phenotypic data from mammalian hybrids derive from qualitative differences in postnatal body size (Gray 1972) and placental phenotypes are rarely collected. Therefore, it is possible that the disruption of hybrid placentation is much more rapidly evolving and widespread than is commonly appreciated. Consistent with this prediction, the rate at which reproductive isolation evolves across different mammal groups has been shown to correlate with physiological aspects of placental morphology (Elliot and Crespi 2006).

The hybrid growth affects noted by (Gray 1972) derive from crosses between species pairs spanning a broad range of taxonomic (intraspecific to intergeneric) and genetic divergence (0 to ~20% pairwise divergence at *cyt b*; Supplemental Table 1). Some of the qualitative growth effects noted by (Gray 1972) are anecdotal and require further validation. Other examples likely reflect heterosis generated through the masking of deleterious recessive alleles and thus do not reflect true intrinsic incompatibilities. In this context, parent-of-origin growth effects likely provide the strongest and most relevant examples of hybrid inviability. The *Phodopus sungorus* × *P. campbelli* cross is especially intriguing because they are among the most closely related species that show asymmetric hybrid growth (3.7% pairwise divergence at *cyt b*). This is on the low end of divergence typically found between sister mammalian species (Bradley and Baker 2001). By comparison, deer mice (*P. polionotus* and *P. maniculatus*; 4.0%), horses and donkeys (7.7%), lions and tigers (11.6%), and house mice (*M. musculus* and *M. spretus*; 9.6%) are all more divergent. Admittedly, mitochondrial DNA often does not accurately reflect genomic divergence between species (Ballard and Whitlock 2004). Nonetheless, our data from hamsters indicate that parent-of-origin growth effects can evolve rapidly and may contribute to the early stages of speciation in mammals.

Our data also support the observation that the evolution of abnormal hybrid growth in mammals tends to follow Haldane's rule. Hybrid mice, hamsters, and deer mice all show male-specific growth phenotypes at some point during development. Deer mouse hybrids show a strongly female-biased sex ratio (Dawson et al. 1993) due to more extreme overgrowth in male placentas and embryos (Vrana et al. 2000; Vrana 2007). Male placentas also tend to be much larger in mouse hybrids between *M. musculus* and *M. spretus* or *M.*

*macedonicus*, (Zechner et al. 1996). Hamster hybrids do not show any sex-specific placental or embryonic differences, but adult cXs hybrid males are severely growth restricted when compared to the females (Figure 2). However, we did observe a weak male-bias in cXs litters (~60%) that could reflect differential female inviability and thus an exception to Haldane's rule. However, we believe that this bias likely results from a maternal effect in this cross. Maternal effects that give rise to skewed sex ratios are common in mammals (Clutton-Brock and Iason 1986) and there were no embryonic phenotypes that indicated females were less viable *in utero*. Given this, a maternal effect seems to be the simplest explanation for this pattern.

#### *Resolving the genetic and epigenetic bases of parent-of-origin growth*

If we assume that abnormal hybrid growth in mammals generally follows the Dobzhansky-Muller model for intrinsic incompatibilities (Dobzhansky 1937; Muller 1942), then it is likely caused by the evolution of incompatible interactions between growth-related genes that have diverged between the hybridizing species. Such failed interactions could disrupt the epigenetic regulation of imprinting and change the expression of genes that control offspring growth. Alternatively, hybrid incompatibilities may cause abnormal growth independent of disrupted imprinting. Differentiating between these two models remains a fundamental problem in mammalian speciation. Epigenetic disruption of imprinting has emerged as the predominant model to explain parent-of-origin dependent growth in mammal hybrids (Vrana 2007; Crespi and Nosil 2013). This model is compelling because errors in imprinting have the ability to explain growth effects (many imprinted genes regulate growth), parent-of-origin effects (imprinting is a parent-of-origin dependent



process), and why these phenotypes are common in mammals (mammals are the only vertebrates where imprinting has been found). Indeed, an analogous regulatory process has been described in the endosperm of angiosperms (Lin 1982; Haig and Westoby 1989), hybrid endosperm development sometimes shows parent-of-origin growth effects (Ishikawa et al. 2011), and disrupted imprinting at endosperm genes has been associated with abnormal endosperm development (Erilova et al. 2009). Nonetheless, despite the broad appeal of this model, no consensus has been reached regarding the role and casual mechanisms of disrupted imprinting on parent-of-origin dependent growth effects in mammals (Zechner et al. 2004; Brown et al. 2012; Wang et al. 2013).

Our data do not support the hypothesis that changes in the imprinting status of our candidate genes cause abnormal hybrid growth in hamsters. Imprinting is maintained at some genes in reciprocal dwarf hamster hybrids while other genes are biallelically expressed in both hybrids (Table 4). Biallelic expression of *Igf2r*, *Grb10*, and *Mash2* may simply reflect that these genes are not imprinted in hamsters. Likewise, variation in the imprinting of *Peg3* (two out of six S×C offspring have biallelic expression) could reflect polymorphism for imprinting of this gene in dwarf hamsters. At this point we cannot distinguish between breakdown and the lack of imprinting at these genes because our crosses relied upon a single outbred strain for each species. However, loss and gain of imprinting does appear to evolve fairly rapidly between species. For example, *Mash2* does not appear to be imprinted in deer mice (Vrana et al. 1998), imprinting of *Igf2r* is polymorphic in humans (Xu et al. 1993), and *Grb10* is imprinted in opposite parental directions in a tissue-specific manner in mice (Garfield et al. 2011). These examples

underscore that much more work is needed to understand the evolutionary rate at which genes gain and/or lose imprinted expression.

The limitations of our expression assay aside, biallelic gene expression in both reciprocal hybrids is unlikely to be directly causal of asymmetric incompatible phenotypes. Monoallelic expression at *Igf2*, *H19*, *Peg3*, and *Snrpn* clearly demonstrates that imprinting is not globally disrupted in placenta of *Phodopus* hybrids. When considered in light of other studies, this result strongly suggests that global loss of imprinting is not common in hybrid mammalian placentas (Vrana et al. 1998; Roemer et al. 1999; Schütt et al. 2003; Wang et al. 2013; but see O'Neill et al. 1998). However, our candidate gene approach does not exclude disrupted imprinting as the ultimate cause of parent-of-origin dependent growth in hamsters. Our experiment has only considered ~10% of the approximately 80 imprinted genes expressed in the mouse placenta (Morison et al. 2005). Imprinted genes also tend to occur in clusters in mammalian genomes (Verona et al. 2003) and cluster-specific imprinting breakdown has been described in deer mice (Wiley et al. 2008). The seven genes that we conclusively surveyed represent only five of the eighteen clusters in house mice (Morison et al. 2005). We are currently collecting genome-wide expression data to determine if cluster-specific breakdown of imprinting also occurs in hybrid dwarf hamsters.

Finally, our data also establish that sex-specific effects are recurrent in the evolution parent-of-origin dependent hybrid growth. At face value this is not surprising given Haldane's rule (Haldane 1922) and the general predictions of dominance theory (Turelli and Orr 2000). However, sex-specific effects are not expected in the placenta because the paternal X chromosome is silenced in extra-embryonic tissues in rodents (imprinted X

chromosome inactivation or XCI; Lyon 1961; 1962). Given imprinted XCI, X-linked genes expressed in the placenta are expected to be effectively hemizygous in both sexes and thus a recessive incompatibility on X chromosome should affect both sexes similarly. Several hypotheses have been proposed to account for unexpected sex-specific effects in hybrid placenta (Hemberger et al. 2001). Recessive X-linked incompatibilities may be partially masked in females due to incomplete silencing of the paternal X chromosome (i.e., leaky imprinted XCI) or through some contribution of X-linked gene products expressed in female embryos where XCI is random (Payer and Lee 2008). Likewise, disruption of imprinted XCI in the placenta could also mask deleterious recessive interactions in females; though it seems unlikely that breakdown of a major epigenetic process would generally result in increased viability. Finally, the male-specific effects could reflect the action of the Y chromosome (Hemberger et al. 2001), though Y-linked effects usually are restricted to male reproductive phenotypes. Differentiating among these potential models will be crucial for resolving the ultimate causes of male-biased developmental abnormalities in mammals.

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## Tables

Table 1. Mammal hybrids with observed growth effects. Phylogenetic independent crosses are in parentheses. Data are from Allen et al. (1993), Dawson (1965), Gray (1972), Sokolov (1993), and Zechner (1996). Details of the exact crosses can be found in Supplemental Table 1.

Order	Reciprocal crosses			Single crosses	
	Both hybrids larger than parent species	Parent of origin growth	Both hybrids smaller than parent species	Larger than parent species	Smaller than parent species
Cetartiodactyl a	5 (3)	1 (1)	-	4 (4)	1 (1)
Carnivora	1 (1)	2 (2)	1 (1)	3 (3)	1 (1)
Perissodactyl a	1 (1)	1 (1)	-	3 (3)	1 (1)
Primates	-	-	-	1 (1)	2 (2)
Rodentia	1 (1)	6 (4)	-	6 (6)	-
Total	8 (5)	10 (8)	1 (1)	17 (17)	5 (5)

Table 2. Late gestation litter size, sex ratio, and developmental defects of dwarf hamsters and their hybrids. Viable embryos are defined as those that were not molar conceptuses (Molar) or reabsorbing (Reab.), but may have edema. Only the viable embryos were used to calculate litter sizes and sex ratios. Significant values are in bold.

Viable Embryos											
Cross	Total	Litters							Litter Size	Females /	Males /
			Females	Males	Unk. Sex	Molar	Reab.	% Male	±se	Litter ±se	Litter ±se
<i>P. campbelli</i>	60	10	NA	NA	58	0	2	n.a.	5.8±0.7	n.a.	n.a.
<i>P. sungorus</i>	52	9	NA	NA	52	0	0	n.a.	5.8±0.5	n.a.	n.a.
<i>P. campbelli</i> × <i>P. sungorus</i>	36	12	13	19	1	0	3	59.3	<b>2.8±0.5<sup>2</sup></b>	1.1±0.3	1.6±0.3
<i>P. sungorus</i> × <i>P. campbelli</i>	73	16	16	18	4	<b>18<sup>1</sup></b>	<b>17<sup>1</sup></b>	52.9	<b>2.4±0.4<sup>2</sup></b>	1.0±0.2	1.1±0.2

<sup>1</sup>*P*<0.001, Fisher’s exact test versus all other cross-types, Bonferroni-corrected α=0.008.

<sup>2</sup>*F*<sub>3,43</sub>= 12.811, *P*<0.001. Also significant in all pairwise Wilcoxon rank-sum tests, *P*<0.0073, Bonferroni-corrected α=0.013.

Table 3. Adult sex ratios in dwarf hamsters and their hybrids partitioned by litter.

Significant values are in bold. Sex ratio was tested using a chi-square test using the pooled sex ratio of the species (50.6% male) as the null expectation.

Cross	Female		% Litters	Male	Litter Size $\pm$ se	Females/Litter $\pm$ se	Males/Litter $\pm$ se
	s	Males					
<i>P. campbelli</i>	199	205	85	50.7	4.8 $\pm$ 0.2	2.3 $\pm$ 0.2	2.4 $\pm$ 0.2
<i>P. sungorus</i>	229	226	77	49.7	5.9 $\pm$ 0.2	3.0 $\pm$ 0.2	2.9 $\pm$ 0.2
<i>P. campbelli</i> $\times$ <i>P.</i> <i>sungorus</i>	60	97	43	<b>61.8<sup>1</sup></b>	<b>3.7<math>\pm</math>0.2<sup>2</sup></b>	1.4 $\pm$ 0.2	2.3 $\pm$ 0.2

<sup>1</sup>Chi-square test,  $X^2=8.463$ ,  $df=1$ ,  $P<0.001$ .

<sup>2</sup> $F_{2,202}=23.665$ ,  $P<0.001$ . Also significant in all pairwise Wilcoxon rank-sum tests,  $P<0.004$ , Bonferroni-corrected  $\alpha=0.025$ .

Table 4. Hybrid expression of eight candidate imprinted genes. Predicted expression is based on the known *M. musculus* expression of these genes described in the Parent-of-Origin Effect Database ([www.otago.ac.nz/IGC](http://www.otago.ac.nz/IGC)). At *Peg3* one SXC male and one female show biallelic expression while all other hybrids show paternal expression.

Gene name	Predicted expression	Hamster hybrid expression	
		cXs	SXC
<i>Grb10</i>	Maternal	Biallelic	Biallelic
<i>H19</i>	Maternal	Maternal	Maternal
<i>Igf2r</i>	Maternal	Biallelic	Biallelic
<i>Mash2</i>	Maternal	Biallelic	Biallelic
<i>Igf2</i>	Paternal	Paternal	Paternal
<i>Mest</i>	Paternal	Not Diagnostic	Not Diagnostic
<i>Peg3</i>	Paternal	Paternal	Polymorphic
<i>Snrpn</i>	Paternal	Paternal	Paternal



## Literature Cited

- Abzhanov, A., M. Protas, B. R. Grant, P. R. Grant, and C. J. Tabin. 2004. *Bmp4* and morphological variation of beaks in Darwin's finches. *Science* 305:1462–1465.
- Allen, W. R. 1969. Factors influencing pregnant mare serum gonadotrophin production. *Nature* 223:64–66.
- Allen, W. R. 2001. Fetomaternal interactions and influences during equine pregnancy. *Reproduction* 121:513–527.
- Allen, W. R., J. A. Skidmore, F. Stewart, and D. F. Antczak. 1993. Effects of fetal genotype and uterine environment on placental development in equids. *J. Reprod. Fertil.* 98:55–60.
- Babak, T., B. DeVeale, C. Armour, C. Raymond, M. A. Cleary, D. van der Kooy, J. M. Johnson, and L. P. Lim. 2008. Global survey of genomic imprinting by transcriptome sequencing. *Curr. Biol.* 18:1735–1741.
- Ballard, J. W. O., and M. C. Whitlock. 2004. The incomplete natural history of mitochondria. *Mol. Ecol.* 13:729–744.
- Bhattacharyya, T., S. Gregorova, O. Mihola, M. Anger, J. Sebestova, P. Denny, P. Simecek, and J. Forejt. 2013. Mechanistic basis of infertility of mouse intersubspecific hybrids. *Proc. Natl. Acad. Sci. B.* 110:E468–E477.
- Boyer, C. C. 1953. Chronology of development for the golden hamster. *J. Morphol.* 92:1–37.
- Bradley, R. D., and R. J. Baker. 2001. A test of the genetic species concept: cytochrome-*b* sequences and mammals. *J. Mammal.* 82:960–973.
- Brideau, N. J., H. A. Flores, J. Wang, and S. Maheshwari. 2006. Two Dobzhansky-Muller genes interact to cause hybrid lethality in *Drosophila*. *Science* 314:1292–1295.
- Brown, J. D., V. Piccuillo, and R. J. O'Neill. 2012. Retroelement demethylation associated

- with abnormal placentation in *Mus musculus* x *Mus caroli* hybrids. *Biol. Reprod.* 86:88–88.
- Burt, A., and R. Trivers. 1998. Genetic conflicts in genomic imprinting. *Proc. Roy. Soc. B.* 265:2393–2397.
- Butler, H., and B. H. J. Juurlink. 1987. An atlas for staging mammalian and chick embryos. CRC Press, Boca Raton, FL.
- Campbell, P., J. M. Good, and M. W. Nachman. 2013. Meiotic sex chromosome inactivation is disrupted in sterile hybrid male house mice. *Genetics* 193:819–828.
- Clutton-Brock, T. H., and G. R. Iason. 1986. Sex ratio variation in mammals. *Q. Rev. Biol.* 61:339–374.
- Cohn, M. J., and C. Tickle. 1999. Developmental basis of limblessness and axial patterning in snakes. *Nature* 399:474–478.
- Coyne, J. A., and H. A. Orr. 1989a. Patterns of speciation in *Drosophila*. *Evolution* 43:362–381.
- Coyne, J. A., and H. A. Orr. 2004. Speciation. Sinauer Associates Inc, Sunderland, MA.
- Coyne, J. A., and H. A. Orr. 1989b. Two rules of speciation. Pp. 180–207 in D. Otte and J. A. Endler, eds. Speciation and its consequences. Sinauer Associates Inc, Sunderland, MA.
- Coyne, J. A., and H. A. Orr. 1997. “Patterns of speciation in *Drosophila*” revisited. *Evolution* 51:295–303.
- Crespi, B. J., and C. Semeniuk. 2004. Parent-offspring conflict in the evolution of vertebrate reproductive mode. *Am. Nat.* 163:635–653.
- Crespi, B. J., and P. Nosil. 2013. Conflictual speciation: species formation via genomic conflict. *Trends Ecol. Evol.* 1:48–57.

- Czech, M. P. 1989. Signal transmission by the insulin-like growth factors. *Cell* 59:235–238.
- Dawson, W. D. 1965. Fertility and size inheritance in a *Peromyscus* species cross. *Evolution* 19:44–55.
- Dawson, W. D., M. N. Sagedy, L. En-yu, D. H. Kass, and J. P. Crossland. 1993. Growth regulation in *Peromyscus* species hybrids: a test for mitochondrial-nuclear genomic interaction. *Growth Dev. Aging*. 57:121–133.
- Dobzhansky, T. G. 1937. *Genetics and the origin of species*. Columbia Univ. Press, New York, NY.
- Drummond, A.J., B. Ashton, M. Cheung, J. Heled, M. Kearse, R. Moir, S. Stones-Havas, T. Thierer, A. Wilson. 2005. Geneious Version 6.1.5 . Available from <http://www.geneious.com/>.
- Duselis, A. R., and P. B. Vrana. 2010. Aberrant growth and pattern formation in *Peromyscus* hybrid placental development. *Biol. Reprod.* 83:988–996.
- Duselis, A. R., and P. B. Vrana. 2007. Assessment and disease comparisons of hybrid developmental defects. *Hum. Mol. Gen.* 16:808–819.
- Elliot, M. G., and B. J. Crespi. 2006. Placental invasiveness mediates the evolution of hybrid inviability in mammals. *Am. Nat.* 168:114–120.
- Erilova, A., L. Brownfield, V. Exner, M. Rosa, D. Twell, O. M. Scheid, L. Hennig, and C. Köhler. 2009. Imprinting of the polycomb group gene MEDEA serves as a ploidy sensor in *Arabidopsis*. *PLOS Genet.* 5:e1000663.
- Felsenstein, J. 2002. PHYLIP (Phylogeny inference package) version 3.6a3. Available from <http://evolution.genetics.washington.edu/phylip.html>.

- Fitzpatrick, B. M. 2004. Rates of evolution of hybrid inviability in birds and mammals. *Evolution* 58:1865.
- Garfield, A. S., M. Cowley, F. M. Smith, K. Moorwood, J. E. Stewart-Cox, K. Gilroy, S. Baker, J. Xia, J. W. Dalley, L. D. Hurst, L. S. Wilkinson, A. R. Isles, and A. Ward. 2011. Distinct physiological and behavioural functions for parental alleles of imprinted *Grb10*. *Nature* 469:534–538.
- Good, J. M., T. Giger, M. D. Dean, and M. W. Nachman. 2010. Widespread over-expression of the X chromosome in sterile F1 hybrid mice. *PLOS Genet.* 6:e1001148.
- Gray, A. P. 1972. Mammalian hybrids. 2nd ed. Commonwealth Agricultural Bureau, Slough, UK.
- Haig, D. 2002. Genomic imprinting and kinship. Rutgers Univ. Press, Piscataway, NJ.
- Haig, D. 1999. Multiple paternity and genomic imprinting. *Genetics* 151:1229–1231.
- Haig, D. 1996. Placental hormones, genomic imprinting, and maternal—fetal communication. *J. Evol. Biol.* 9:357–380.
- Haig, D., and C. Graham. 1991. Genomic imprinting and the strange case of the insulin-like growth factor II receptor. *Cell* 64:1045.
- Haig, D., and M. Westoby. 1989. Parent-specific gene expression and the triploid endosperm. *Am. Nat.* 134:147–155.
- Haldane, J. B. S. 1922. Sex ratio and unisexual sterility in hybrid animals. *J. Genet.* 12:101–109.
- Hemberger, M. C., R. S. Pearsall, U. Zechner, A. Orth, S. Otto, F. Rüschen-dorf, R. H. Fundele, and R. W. Elliott. 1999. Genetic dissection of X-linked interspecific hybrid placental dysplasia in congenic mouse strains. *Genetics* 153:383–390.

- Hemberger, M., H. Kurz, A. Orth, S. Otto, A. Lüttges, R. W. Elliott, A. Nagy, S.-S. Tan, P. Tam, U. Zechner, and R. H. Fundele. 2001. Genetic and developmental analysis of X-inactivation in interspecific hybrid mice suggests a role for the Y chromosome in placental dysplasia. *Genetics* 157:341–348.
- Ishikawa, R., T. Ohnishi, Y. Kinoshita, M. Eiguchi, N. Kurata, and T. Kinoshita. 2011. Rice interspecies hybrids show precocious or delayed developmental transitions in the endosperm without change to the rate of syncytial nuclear division. *Plant J.* 65:798–806.
- Kurz, H., U. Zechner, A. Orth, and R. H. Fundele. 1999. Lack of correlation between placenta and offspring size in mouse interspecific crosses. *Anat. Embryol.* 200:335–343.
- Leiser, R., and P. Kaufmann. 1994. Placental structure: in a comparative aspect. *Exp. Clin. Endocrinol.* 102:122–134.
- Li, L. L., E. B. Keverne, S. A. Aparicio, F. Ishino, and S. C. Barton. 1999. Regulation of maternal behavior and offspring growth by paternally expressed *Peg3*. *Science* 284:330–333.
- Lin, B. Y. 1982. Association of endosperm reduction with parental imprinting in maize. *Genetics* 100:475–486.
- Lindor, N. M., J. A. Ney, T. A. Gaffey, I. Jenkins, S. N. Thibodeau, and G. W. DeWald. 1992. A genetic review of complete and partial hydatidiform moles and nonmolar triploidy. *Mayo Clin. Proc.* 67:791–799.
- Loschiavo, M., Q. K. Nguyen, A. R. Duselis, and P. B. Vrana. 2007. Mapping and identification of candidate loci responsible for *Peromyscus* hybrid overgrowth. *Mamm. Genome* 18:75–85.
- Lyon, M. F. 1961. Gene action in the X-chromosome of the mouse (*Mus musculus* L.). *Nature*

190:372–373.

Lyon, M. F. 1962. Sex chromatin and gene action in the mammalian X-chromosome. *Am. J. Hum. Genet.* 14:135–148.

Mallarino, R., O. Campàs, J. A. Fritz, K. J. Burns, O. G. Weeks, M. P. Brenner, and A. Abzhanov. 2012. Closely related bird species demonstrate flexibility between beak morphology and underlying developmental programs. *Proc. Natl. Acad. Sci. B.* 109:16222–16227.

Masly, J. P., and D. C. Presgraves. 2007. High-resolution genome-wide dissection of the two rules of speciation in *Drosophila*. *PLOS Biol.* 5:1890–1898.

Meiklejohn, C. D., E. L. Landeen, J. M. Cook, S. B. Kingan, and D. C. Presgraves. 2011. Sex chromosome-specific regulation in the *Drosophila* male germline but little evidence for chromosomal dosage compensation or meiotic inactivation. *PLOS Biol.* 9:e1001126.

Morison, I. M., J. P. Ramsay, and H. G. Spencer. 2005. A census of mammalian imprinting. *Trends Genet.* 21:457–465.

Muller, H. J. 1942. Isolating mechanisms, evolution, and temperature. *Biol. Symp.* 6:71–125.

Neumann, K., J. Michaux, V. Lebedev, N. Yigit, E. Colak, N. Ivanova, A. Poltoraus, A. Surov, G. Markov, S. Maak, S. Neumann, and R. Gattermann. 2006. Molecular phylogeny of the Cricetinae subfamily based on the mitochondrial cytochrome *b* and 12S rRNA genes and the nuclear vWF gene. *Mol. Phylogenet. and Evol.* 39:135–148.

Newkirk, K. D., H. J. McMillan, and K. E. Wynne-Edwards. 1997. Length of delay to birth of a second litter in dwarf hamsters (*Phodopus*): Evidence for post-implantation embryonic diapause. *J. Exp. Zool.* 278:106–114.

O'Neill, R. J. W., M. J. O'Neill, and J. A. M. Graves. 1998. Undermethylation associated with

- retroelement activation and chromosome remodelling in an interspecific mammalian hybrid. *Nature* 393:68–72.
- Orr, H. A. 1996. Dobzhansky, Bateson, and the genetics of speciation. *Genetics* 144:1331–1335.
- Payer, B., and J. T. Lee. 2008. X chromosome dosage compensation: how mammals keep the balance. *Annu. Rev. Genet.* 42:733–772.
- Piedrahita, J. A. 2011. The role of imprinted genes in fetal growth abnormalities. *Birth Defects Res. A. Clin. Mol. Teratol.* 91:682–692.
- Prager, E. M., and A. C. Wilson. 1975. Slow evolutionary loss of the potential for interspecific hybridization in birds: a manifestation of slow regulatory evolution. *Proc. Natl. Acad. Sci. B.* 72:200–204.
- R Core Team. 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL: <http://www.R-project.org/>.
- Reik, W., M. Constancia, A. Fowden, N. Anderson, W. Dean, A. Ferguson-Smith, B. Tycko, and C. Sibley. 2003. Regulation of supply and demand for maternal nutrients in mammals by imprinted genes. *J. Physiol.* 547:35–44.
- Roemer, I., F. Grutzner, H. Winking, T. Haaf, and A. Orth. 1999. Genome evolution: global methylation in eutherian hybrids. *Nature* 401:131–132.
- Rogers, J. F., and W. D. Dawson. 1970. Foetal and placental size in a *Peromyscus* species cross. *J. Reprod. Fertil.* 21:255–262.
- Ross, P. D. 1995. *Phodopus campbelli*. *Mamm. Species* 503:1–7.
- Ross, P. D. 1998. *Phodopus sungorus*. *Mamm. Species* 595:1–9.
- Rozen, S., and H. Skaletsky. 2000. Primer3 on the WWW for general users and for biologist

- programmers. Pp. 365–386 in S. Misener and S. A. Krawetz, eds. *Bioinformatics Methods and Protocols: Methods for Molecular Biology*. Humana Press, Totowa, NJ.
- Safronova, L. D., and N. Y. Vasil'eva. 1996. Meiotic Abnormalities in Interspecific Hybrids between *Phodopus sungorus* (Pallas, 1773) and *Ph. campbelli* (Thomas, 1905). *Russ. J. Genet.* 32:486–494.
- Safronova, L. D., E. V. Cherepanova, and N. Y. Vasil'eva. 1999. Specific features of the first meiotic division in hamster hybrids obtained by backcrossing *Phodopus sungorus* and *Phodopus campbelli*. *Russ. J. Genet.* 35:184–188.
- Saukkonen, T. 2004. Dose-Dependent Effects of Recombinant Human Insulin-Like Growth Factor (IGF)-I/IGF Binding Protein-3 Complex on Overnight Growth Hormone Secretion and Insulin Sensitivity in Type 1 Diabetes. *J. Clin. Endocr. Metab.* 89:4634–4641.
- Schütt, S., A. R. Florl, W. Shi, M. Hemberger, and A. Orth. 2003. DNA methylation in placentas of interspecies mouse hybrids. *Genetics* 165:223–228.
- Scribner, S. J., and K. E. Wynne-Edwards. 1994. Disruption of body temperature and behavior rhythms during reproduction in dwarf hamsters ( *Phodopus*). *Physiol. Behav.* 55:361–369.
- Shapiro, M. D., M. E. Marks, C. L. Peichel, B. K. Blackman, K. S. Nereng, B. Jónsson, D. Schluter, and D. M. Kingsley. 2004. Genetic and developmental basis of evolutionary pelvic reduction in threespine sticklebacks. *Nature* 428:717–723.
- Shaw, C. N., P. J. Wilson, and B. N. White. 2003. A reliable molecular method of gender determination for mammals. *J. Mammal.* 84:123–128.
- Shi, W., A. Krella, A. Orth, Y. Yu, and R. H. Fundele. 2005. Widespread disruption of genomic



- imprinting in adult interspecies mouse (*Mus*) hybrids. *genesis* 43:100–108.
- Shi, W., L. Lefebvre, Y. Yu, S. Otto, A. Krella, A. Orth, and R. H. Fundele. 2004. Loss-of-imprinting of *Peg1* in mouse interspecies hybrids is correlated with altered growth. *genesis* 39:65–72.
- Sobel, J. M., G. F. Chen, L. R. Watt, and D. W. Schemske. 2010. The biology of speciation. *Evolution* 64:295–315.
- Sokolov, V. E., and N. I. Vasil'eva. 1993. Hybridologic analysis confirms the species specificity of *Phodopus sungorus* (Pallus, 1773) and *Phodopus campbelli* (Thomas, 1905). *Dokl. Akad. Nauk.* 332:120–123.
- Surani, M. A., N. D. Allen, S. C. Barton, R. H. Fundele, S. K. Howlett, M. L. Norris, and W. Reik. 1990. Developmental consequences of imprinting of parental chromosomes by DNA methylation. *Philos. Trans. R. Soc. London. B.* 326:313–327.
- Tang, S., and D. C. Presgraves. 2009. Evolution of the *Drosophila* nuclear pore complex results in multiple hybrid incompatibilities. *Science* 323:779–782.
- Theiler, K. 1972. The house mouse. Development and normal stages from fertilization to 4 weeks of age. Springer-Verlag, New York, NY.
- Turelli, M., and H. A. Orr. 2000. Dominance, epistasis and the genetics of postzygotic isolation. *Genetics* 154:1663–1679.
- Turelli, M., and H. A. Orr. 1995. The dominance theory of Haldane's rule. *Genetics* 140:389–402.
- Varmuza, S. 1993. Gametic imprinting as a speciation mechanism in mammals. *J. Theor. Biol.* 164:1–13.
- Verona, R. I., M. R. W. Mann, and M. S. Bartolomei. 2003. Genomic imprinting: intricacies of

- epigenetic regulation in clusters. *Annu. Rev. Cell Dev. Biol.* 19:237–259.
- Vrana, P. B. 2007. Genomic imprinting as a mechanism of reproductive isolation in mammals. *J. Mammal.* 88:5–23.
- Vrana, P. B., J. A. Fossella, P. Matteson, T. del Rio, M. J. O'Neill, and S. M. Tilghman. 2000. Genetic and epigenetic incompatibilities underlie hybrid dysgenesis in *Peromyscus*. *Nat. Genet.* 25:120–124.
- Vrana, P. B., X. J. Guan, R. S. Ingram, and S. M. Tilghman. 1998. Genomic imprinting is disrupted in interspecific *Peromyscus* hybrids. *Nat. Genet.* 20:362–365.
- Wang, X., D. C. Miller, and R. Harman. 2013. Paternally expressed genes predominate in the placenta. *Proc. Natl. Acad. Sci. B.* 110:10705–10710.
- West, J. D., W. I. Frels, and V. E. Papaioannou. 1977. Development of interspecific hybrids of *Mus*. *J. Embryol. Exp. Morph.* 41:233–243.
- Wiley, C. D., H. H. Matundan, A. R. Duselis, A. T. Isaacs, and P. B. Vrana. 2008. Patterns of hybrid loss of imprinting reveal tissue- and cluster-specific regulation. *PLOS ONE* 3:e3572.
- Wright, S. 1921. Systems of mating. II. The effects of inbreeding on the genetic composition of a population. *Genetics* 6:124–143.
- Xu, Y., C. G. Goodyer, C. Deal, and C. Polychronakos. 1993. Functional polymorphism in the parental imprinting of the human *IGF2R* gene. *Biochem. Biophys. Res. Comm.* 197:747–754.
- Zechner, U., M. Reule, A. Orth, F. Bonhomme, B. Strack, J.-L. Guénet, H. Hameister, and R. H. Fundele. 1996. An X-chromosome linked locus contributes to abnormal placental development in mouse interspecific hybrids. *Nat. Genet.* 12:398–403.

Zechner, U., M. Reule, P. S. Burgoyne, A. Schubert, A. Orth, H. Hameister, and R. H. Fundele.

1997. Paternal transmission of X-linked placental dysplasia in mouse interspecific hybrids. *Genetics* 146:1399–1405.

Zechner, U., W. Shi, M. Hemberger, H. Himmelbauer, S. Otto, A. Orth, V. Kalscheuer, U.

Fischer, R. Elango, A. Reis, W. Vogel, H. Ropers, F. R schendorf, and R. H. Fundele.

2004. Divergent genetic and epigenetic post-zygotic isolation mechanisms in *Mus* and *Peromyscus*. *J. Evol. Biol.* 17:453–460.

Zeh, D. W., and J. A. Zeh. 2000. Reproductive mode and speciation: the viviparity-driven conflict hypothesis. *Bioessays* 22:938–946.

## Figure Legends

Figure 1. Average weights ( $\pm 2$  SE) of late-term embryos (A) and placentas (B). Letters designate significant differences between groups based on pairwise Wilcoxon rank-sum tests and group sample sizes are in parentheses. Insets show (C) a normal *P. campbelli* offspring with average sized placenta and (D) an overgrown *P. sungorus*  $\times$  *P. campbelli* (S $\times$ C) offspring with an enlarged placenta.

Figure 2. Growth curves for *P. campbelli*, *P. sungorus*, and *P. campbelli*  $\times$  *P. sungorus* (c $\times$ s) offspring. Average weights ( $\pm 2$  SE) are shown every 10 days.

Figure 3. Average weights ( $\pm 2$  SE) of hamsters at 80 days. Filled circles represent females, empty circles represent males, and sample sizes are given in parentheses. \*\* $P < 0.001$ , \* $P < 0.05$ , pairwise *t*-test, Bonferroni corrected  $\alpha = 0.017$ .

Figure 1

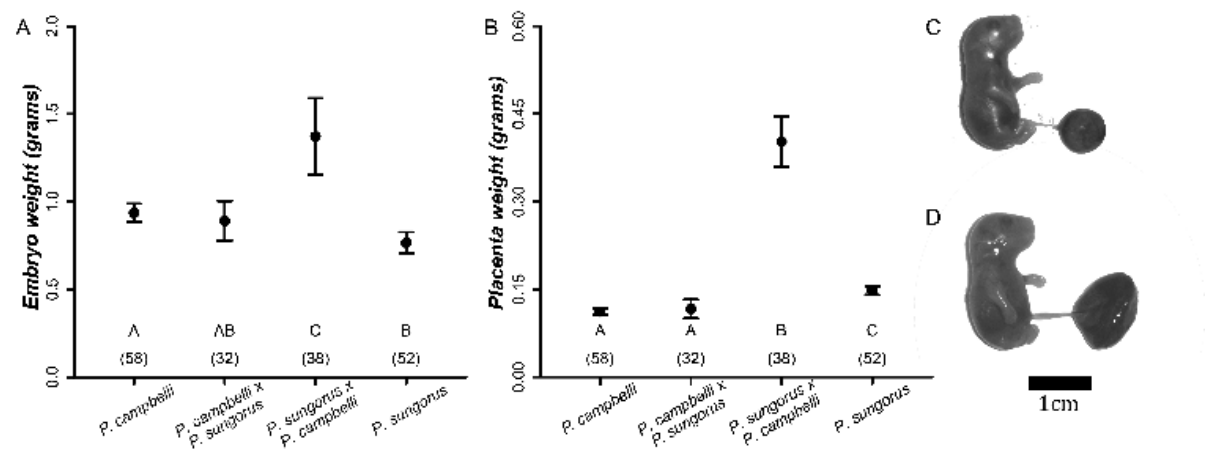


Figure 2

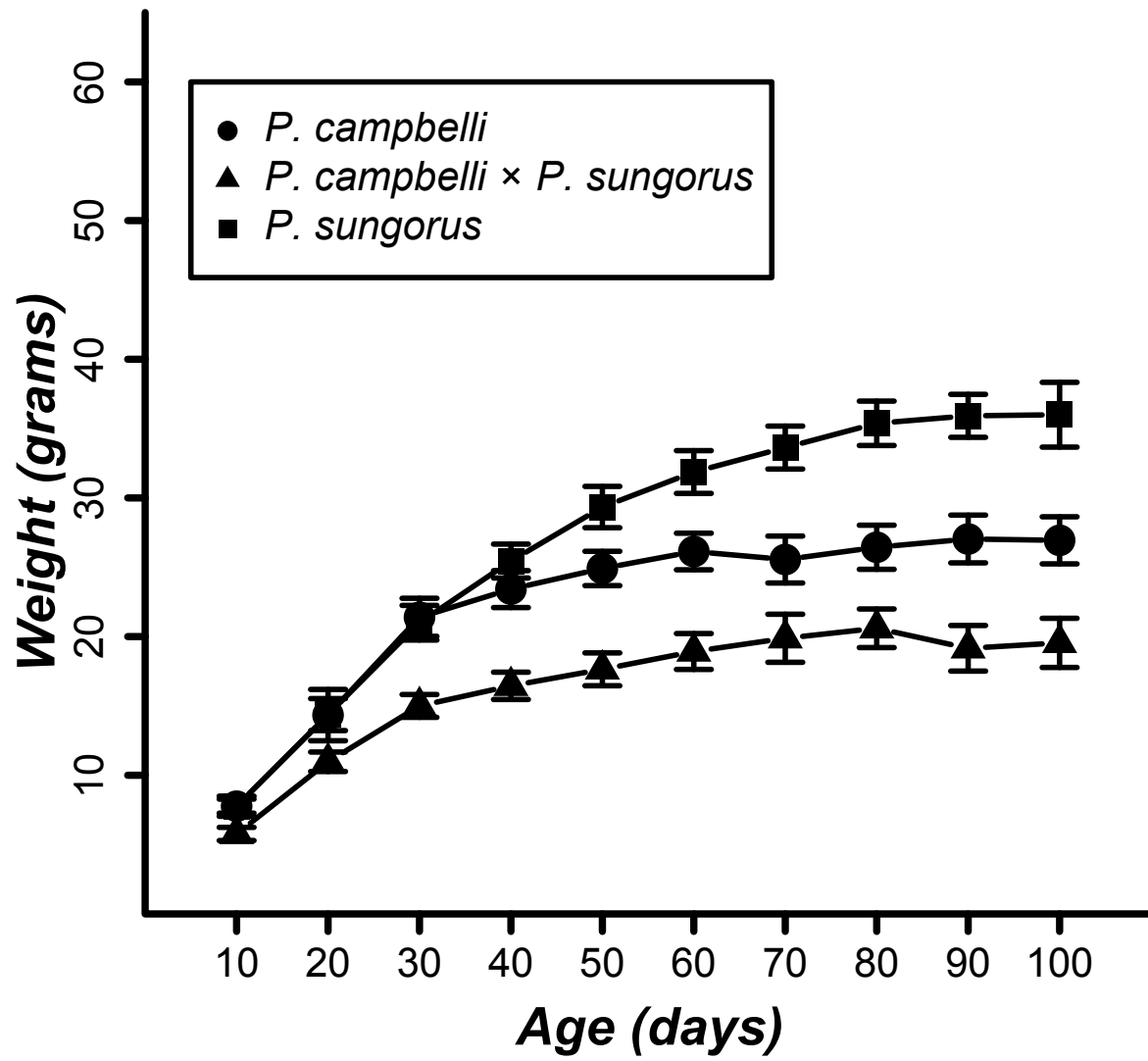


Figure 3

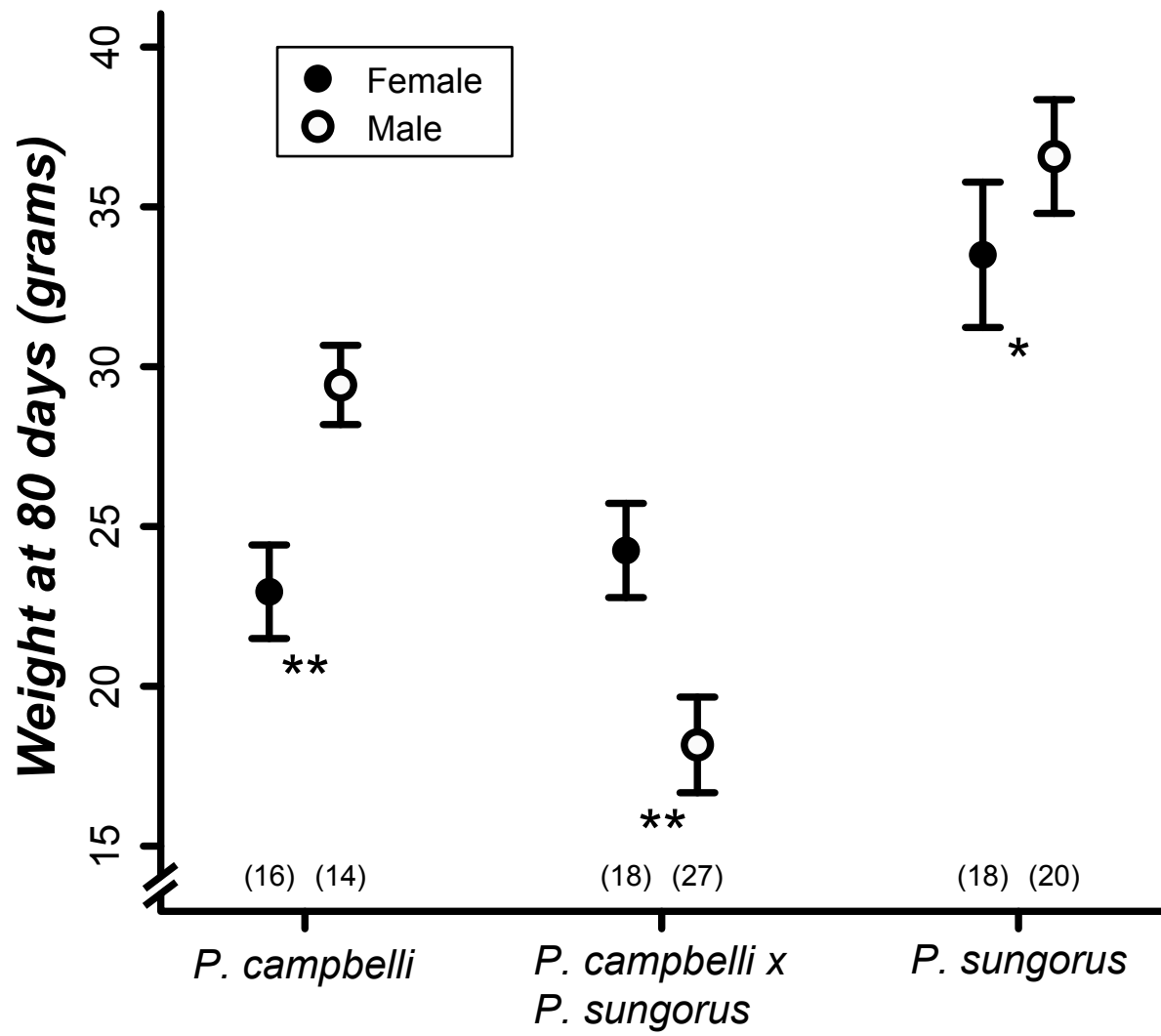
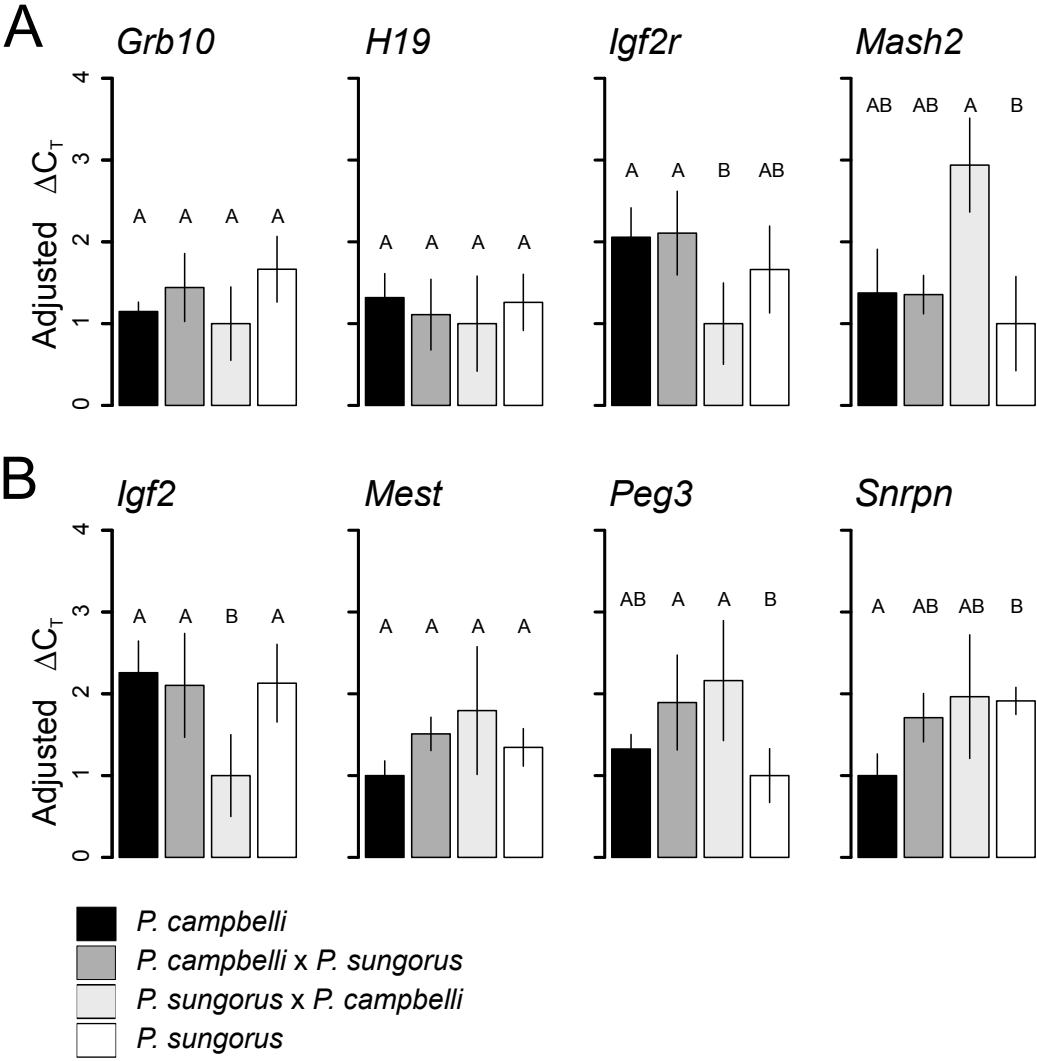
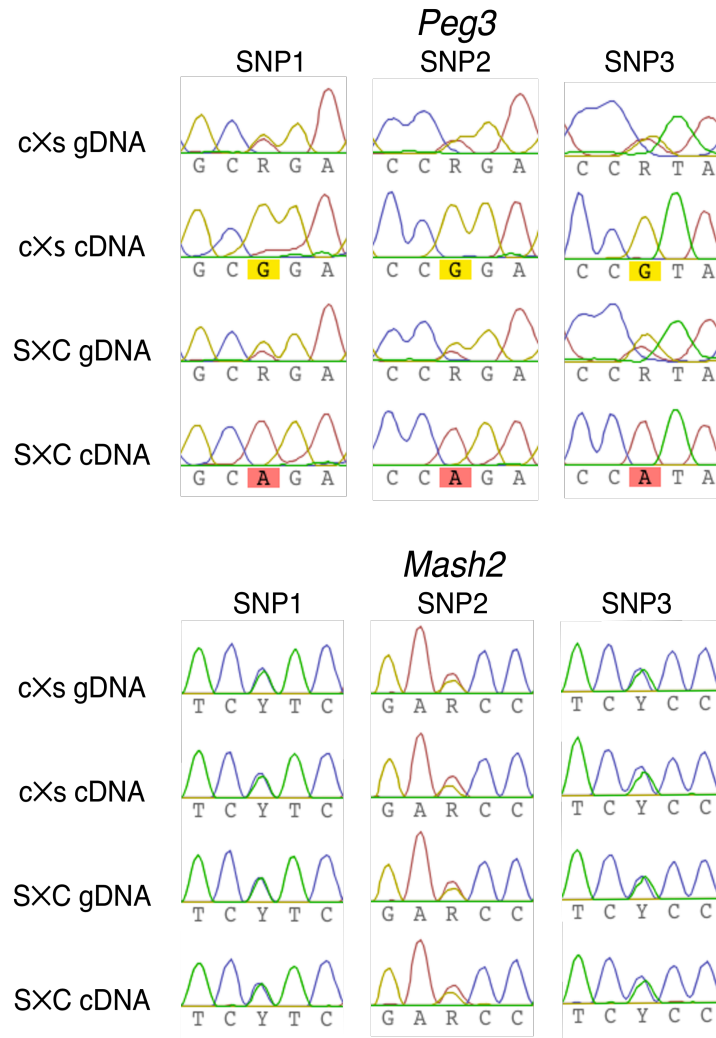


Figure 4



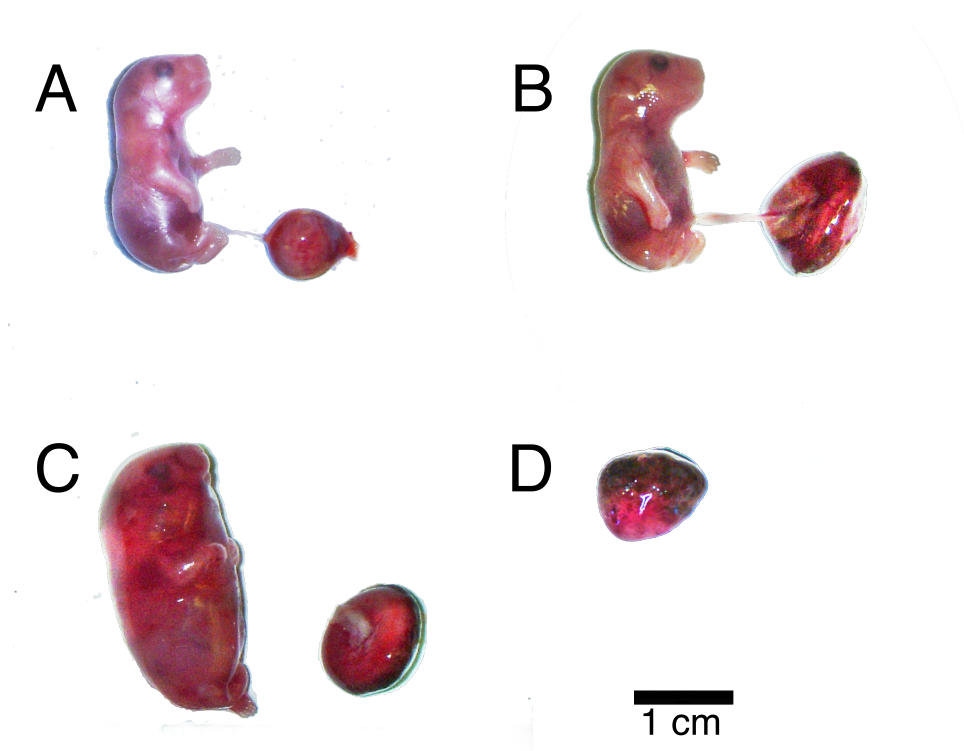


Supplemental Figure 1



Supplemental Figure 1: Examples of a gene that is properly imprinted in reciprocal hybrids (*Peg3*, top) and one that shows biallelic expression in hybrids (*Mash2*, bottom). Genomic DNA (gDNA) and complement DNA (cDNA) from the same individual demonstrate heterozygosity at three fixed differences in the gDNA sequences for both of these genes. In contrast, when cDNA is sequenced, these heterozygous positions show imprinted expression of *Peg3* and biallelic expression of *Mash2*.

Supplemental Figure 2



Supplemental Figure 2: (A) Normal *P. campbelli* offspring with average sized placenta. (B) Overgrown SXC offspring with an enlarged placenta. (C) Overgrown SXC offspring with severe edema (swelling) of the body and an enlarged placenta. (D) Overgrown SXC molar conceptus. All images are to scale.



## Supplemental Table 2

Table S2: PCR Primer Sequences and Reaction Conditions

Primer Name	Sequence 5' to 3'	Melting Temp (T <sub>m</sub> )	GenBank accession numbers or USCS gene IDs/genome builds used to design these primer pairs
<i>Cytb</i> Forward	CCWGCCCATCAAAATYTC	60	
<i>Cytb</i> Reverse	ACTGGTTGNCTCCRAITCA	60	UCSC: uc009kob.1_mm9, m4
<i>Grb10</i> Forward	GCCTTCAGGAGGAGACCA	55	
<i>Grb10</i> Reverse	CATGGAACCACTGTCTNTIC	55	UCSC: NM_001001555_hg18, mm9, m4, cavPor2
<i>H19</i> Forward	GACATGGTCCGGGTGAVG	55	
<i>H19</i> Reverse	CTGGTGRGGAGGGGCAAA	55	UCSC: uc009kob.1_mm9, hg19, m4
<i>Igf2</i> Forward	TGGGGAAGTCGATGTTGG	55	
<i>Igf2</i> Reverse	CGYTTGGCTCTCTGACAC	55	UCSC: uc009kod.1_mm9, hg19, m4, cavPor3
<i>Igf2r</i> Forward	ACCACGAGTGGGGCTTCT	59	
<i>Igf2r</i> Reverse	GCCACGAGGAGGCTGAG	59	UCSC: uc008aky.1_mm9, hg18, m4, cavPor2
<i>Mash2</i> Forward	GAGCGCAACCGCTRAAG	57	
<i>Mash2</i> Reverse	TCAGTAGCCCTCAACACACTG	57	UCSC: uc009koj.1_mm9, hg18, m4, cavPor2
<i>Mest</i> Forward	GAGRGATGGTGGTCCARG	56	
<i>Mest</i> Reverse	AAGGAGTTGATGAAGCCATA	56	UCSC: uc009bfu.1_mm9, hg18, cavPor2
<i>Peg3</i> Forward	TGTGGACAGGCTTCATTCA	55	
<i>Peg3</i> Reverse	TGTGAGAATCTGGTGCTGG	55	UCSC: NM_001146186_mm9, hg18, m4, cavPor2
<i>Sorpn</i> Forward	TGTGGGTAAGAGTAGCAAGATGC	55	
<i>Sorpn</i> Reverse	GTCTTGGTGGRCGCTTC	55	UCSC: NM_022807_mm9, m4
<i>Zfy</i> Forward*	CAAAWCTGCAAGGRTAGAC	60	
<i>Zfy</i> Reverse*	AGACTGATTCAGGAGTAGCA	60	GenBank: X75172.1, X75171.1, NM_001044386.1, AY012058.1, M74776.1, AY012055.1
<i>Grb10_sper</i> Gen_F	CAGGTGAAGGAAGTTGGAAG	60	
<i>Grb10_sper</i> Gen_R	GGACTTTGCCACGAAGGAA	60	GenBank: JX217835.1, JX217834.1
<i>H19_sper</i> F1	TGTTCTCTCAAGCAAGAA	60	
<i>H19_sper</i> R1	CGTCACTCTCTCTGTCTT	60	GenBank: JX217837.1, JX217836.1
<i>Igf2_sper</i> F1	GAGGCACTGTGGAAGAGTG	60	
<i>Igf2_sper</i> R1	ACACGTCCTCTCGGACTT	60	GenBank: JX217838.1, JX217839.1
<i>Igf2r_sper</i> F2	AATGACCAAGCACTTCAGCAG	60	
<i>Igf2r_sper</i> R2	TGGAAGAAGATGGTGATAGA	60	GenBank: JX 217841.1, JX217840.1
<i>Mash2_sper</i> F2	CGTTATCTCTCCGCAAGT	60	
<i>Mash2_sper</i> R2	CACCGCACTCAGCTCTCC	60	GenBank: JX217843.1, JX217842.1
<i>Mest_sper</i> F1	GCTTTGGCTTCAGTGACAAA	60	
<i>Mest_sper</i> R1	TGATTCGCGGTTCTGTAGC	60	GenBank: JX217845.1, JX217844.1
<i>Peg3_sper</i> F1	CAGATGGAAGCTGCTGAG	60	
<i>Peg3_sper</i> R2	CTTTTCTGGGTTCTCGATCC	60	GenBank: JX217847.1, JX217846.1
<i>Sorpn_sper</i> F1	GGAGGGTCCACCTCTAAAG	60	
<i>Sorpn_sper</i> R1	GGACAGGACTCTGTAATCCA	60	GenBank: JX217849.1, JX217848.1
<i>Ywhaz_sper</i> F1	GCCTGCTCTCTGCAAAAC	60	
<i>Ywhaz_sper</i> R2	ATTTTCCCTCTCTCTCTG	60	CHO-K1 (GCA_000223135.1)

PCR reaction conditions: 2min at 94c, 30x(15sec at 94c, 15sec at T<sub>m</sub>, 60sec at 72c), 60sec at 72c, hold at 10c  
 qPCR reaction conditions: 10min at 95c, 40x(30sec at 95c, 15sec at T<sub>m</sub>, 15sec at 72c), 1min at 95c, 30sec at 55c, 30sec at 95c

\*This primer pair is similar to LGL331 and LGL335 from Shaw (2003) but have some slight modifications that result in them not amplifying *Zfy* in hamsters. We have therefore chosen to name them differently despite their similarities and common origin.

Shaw, C. N., P. J. Wilson, and B. N. White. 2003. A reliable molecular method of gender determination for mammals. *J. Mammal.* 84:123–128.

# **DISRUPTED PLACENTAL EXPRESSION, GENOMIC IMPRINTING, AND SPECIATION IN DWARF HAMSTERS**

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## Abstract

The importance of gene regulatory incompatibilities to the early stages of speciation remains unclear. In mammals, extreme hybrid growth is common and often shows a parent-of-origin effect where reciprocal hybrids differ in size and are either larger or smaller than the parental species. Disruption of genomic imprinting, the parent-specific epigenetic silencing (imprinting) of one allele, has been linked to diverse developmental diseases and has been hypothesized to be the predominant cause of abnormal hybrid growth. This hypothesis predicts that loss of placental imprinting in hybrids results in parent-of-origin dosage imbalances between paternally expressed growth factors and maternally expressed repressors that in turn cause abnormal embryonic and placental growth. Here we test the general predictions of this model by dissecting patterns of placental gene expression in a reciprocal cross between two species of dwarf hamsters (*Phodopus sungorus* and *P. campbelli*) that shows extreme parent-of-origin hybrid growth. In hybrids with massively enlarged placentas we observed both extensive transgressive expression of growth-related genes and bi-allelic expression of a large set of genes that normally show paternal silencing. However, the apparent widespread disruption of paternal imprinting was strongly coupled with significantly reduced gene expression levels overall. These patterns are contrary to the predictions of the loss of imprinting model and indicate that hybrid misexpression of dosage sensitive genes is caused by other mechanisms in this system. Collectively, our results support a central role for disrupted gene expression in mammalian speciation, but call in to question the generality of the widely accepted loss of genomic imprinting model.

## **Author Summary**

Hybridization in mammals often results in offspring of unusual size, suggesting that regulatory evolution plays an important role in the origin of species. Partial loss of a key epigenetic phenomenon — genomic imprinting — has often been invoked to explain patterns of abnormal growth in both human diseases and hybrid mammals. Genomic imprinting is the parent-specific silencing of one allele at genes that are often involved in mammalian development. Here we test if genomic imprinting specifically, and gene expression generally, is disrupted in the placentas of hybrids that show extreme growth during the later stages of pregnancy. We find strong evidence for widespread disruptions in levels and allelic usage of gene expression in overgrown hybrids, but no evidence for loss of genomic imprinting. Our study provides a clear link between abnormal development, disrupted gene expression, and speciation but underscores that the mechanisms underlying these processes are likely to be diverse.

## Introduction

Gene expression plays a central role in organismal development and morphological evolution (Cohn and Tickle 1999; Abzhinov et al. 2004; Mallarino et al. 2012; Shapiro et al. 2004), but the importance of regulatory divergence to speciation remains unclear (Butlin et al. 2012; Wolf, Lindell, and Backstrom 2010; Prud'homme, Gompel, and Carroll 2007).

Mammalian hybrids often show extreme parent-of-origin growth effects where reciprocal hybrids differ in size and are either much larger or smaller than the parental species (Vrana 2007; Brekke and Good 2014). Parent-of-origin dependent hybrid growth typically manifests in the placenta and embryo during developmental stages that are known to be highly dosage sensitive (Moore and Haig 1991; Haig 1996; Crespi and Semeniuk 2004; Reik et al. 2003), suggesting that this often severe form of hybrid inviability may be a consequence of regulatory incompatibilities. However, it is unclear if the recurrent evolution of this general class of hybrid phenotypes reflects a common regulatory and genetic basis.

Genomic imprinting is a form of gene regulation that involves the parent-specific epigenetic silencing (imprinting) of one allele (Morison, Ramsay, and Spencer 2005). Imprinted genes are often involved in embryonic growth and development and the disruption of genomic imprinting has been hypothesized to be the predominant cause of abnormal hybrid growth in mammals (Vrana 2007). This general model is based on three related observations. First, placental growth pathways are highly enriched for imprinted genes that show strong parent-of-origin functional associations. In general, paternally expressed genes tend to promote growth, while maternally expressed genes tend to act as



growth repressors (Haig 1996; Reik et al. 2003; Saukkonen 2004). Second, the evolution of imprinting is intimately linked to parental conflict (Trivers 1974). While there are many theoretical explanations for the evolution of genomic imprinting (Haig and Trivers 1995; Solter 1988; Sapienza 1989; Hall 1990; Barlow 1993; Varmuza and Mann 1994; Wolf and Hager 2006; Haig 2000; Moore and Haig 1991; Spencer and Clark 2014), the functionally antagonist roles of alternatively imprinted genes are nonetheless predicted to result in genetic conflict and rapid evolutionary divergence (Haig 1996). Third, the disruption of genomic imprinting has been linked to many growth-related diseases in humans and other mammals, including various cancers and developmental syndromes (Lim and Maher 2010; Bjornsson et al. 2007; Kishino, Lalande, and Wagstaff 1997; Nicholls and Knepper 2001; Buiting et al. 1995; Reik and Maher 1997; Weksberg, Shuman, and Beckwith 2010; Eggermann et al. 2006; Z. Chen et al. 2015).

Imprinted expression of a gene is often regulated through allele-specific methylation of promoter DNA or histones, or through the expression of an antisense long non-coding RNA (Ideraabdullah, Vigneau, and Bartolomei 2008; J. R. Mann et al. 2000). DNA Methylation typically acts as a repressive mark and thus hypomethylation of an imprinted gene may activate the expression of a silenced allele in what is termed loss-of-imprinting (LOI). Though there are several potential ways that imprinting could contribute to hybrid incompatibilities (Brekke and Good 2014; Chakraborty 1989; Varmuza 1993), the speciation literature has primarily emphasized loss of DNA methylation and associated LOI in hybrids (Crespi and Nosil 2013; Vrana 2007; Brekke and Good 2014; Shi et al. 2005; Brown, Piccuillo, and O'Neill 2012; Vrana et al. 1998; Vrana et al. 2000). Within imprinted growth pathways, LOI is predicted to result in parent-of-origin dependent dosage

imbalances between paternally-expressed growth factors and maternally-expressed repressors. Specifically, failure of maternal silencing is expected increase the expression of growth-promoting genes and result in offspring overgrowth, while the failure of paternal silencing increases the dose of growth repressors, retarding offspring growth (Brekke and Good 2014; Vrana 2007).

The predictions of LOI are fairly straightforward, but empirical support for this model has been mixed. To date, LOI and the expression of imprinted genes has been evaluated in the hybrids of house mice (Zechner et al. 1996; Zechner et al. 1997; Kurz et al. 1999; Zechner et al. 2002; Schütt et al. 2003; Ishikawa et al. 2003; Zechner et al. 2004; Gregg, Zhang, Weissbourd, et al. 2010; Gregg, Zhang, Butler, et al. 2010; Xu Wang, Soloway, and Clark 2011), deer mice (Vrana et al. 1998; Vrana et al. 2000; Vrana et al. 2001; Duselis et al. 2005; Duselis and Vrana 2007; Duselis et al. 2007; Loschiavo et al. 2007; Vrana 2007; Duselis and Vrana 2010; Wiley et al. 2008), horses and donkeys (Xu Wang et al. 2013), dwarf hamsters (Brekke and Good 2014), and cows (Z. Chen et al. 2015; Z. Chen et al. 2014). There are many cases where patterns of expression at specific genes in various hybrids support the LOI model. Examples including *Peg1* (*Mest*) and *Snrpn* in house mice (Shi et al. 2004; Shi et al. 2005), and *Peg3* and *Peg10* in deer mice (Vrana et al. 1998; Wiley et al. 2008), all of which show the expected correlation between LOI, expression level, gene function, and hybrid size. On the other hand, many genes fail to meet at least one of the predictions of the dosage model. For instance, in house mice the paternally-expressed growth promoter *Peg3*, unexpectedly shows LOI in the skeletal muscle of small hybrids (Shi et al. 2005), and in deer mice, *Dcn*, a maternally-expressed growth repressor, shows LOI in large hybrids (Wiley et al. 2008). Conflicting evidence can also be found in cattle displaying

abnormal growth (Z. Chen et al. 2015) and there is no evidence for LOI in hybrids between horses and donkeys (Xu Wang et al. 2013) despite the observed growth effects in mules and hinnies (Allen et al. 2004). In sum, the LOI model shows mixed support among studies primarily focused on a small set of candidate genes (but see (Z. Chen et al. 2015; Xu Wang et al. 2013)), and the generality of the LOI model remains unclear.

Here we use genome-wide approaches to dissect the regulatory underpinnings of extreme placental overgrowth that manifests in the hybrids of two closely related dwarf hamster species. Reciprocal crosses between *Phodopus sungorus* and *P. campbelli* result in strong parent-of-origin effects for placental and embryonic growth (Brekke and Good 2014). F1 hybrids derived from a *P. sungorus* female crossed to a *P. campbelli* male (hereafter “S×C”) show massive placental and embryonic overgrowth and a range of associated birth defects, usually resulting in late-term failure of hybrid pregnancies and maternal death (Brekke and Good 2014; Safronova and Vasil'eva 1996; Safronova, Cherepanova, and Vasil'eva 1999; Sokolov and Vasil'eva 1993). Reciprocal hybrids (hereafter “c×s”) appear phenotypically normal *in utero*, though adult c×s males are significantly smaller than males from either species (Brekke and Good 2014). We compare the late-term placental histology and genome-wide patterns of placental expression between both species and their reciprocal hybrids to determine if abnormal hybrid growth is associated with disrupted expression. Using patterns of allele-specific expression (ASE), we systematically test the central predictions of the LOI model. Collectively, our data provide several novel insights into the evolution of genomic imprinting and the likely causes and consequences of regulatory incompatibilities in hybrid mammals.

## Results

### *Similar placental histology between species and reciprocal hybrids*

Histology needs to be carefully considered when interpreting tissue-level gene expression data because underlying changes in cellular composition can result in spurious signals of differential expression, especially for genes with cell-specific expression (Good et al. 2010). This cellular composition effect can be particularly strong when considering extreme tissue phenotypes. The placenta is a complex tissue including two purely fetal cell layers (labyrinthine trophoblast and spongiotrophoblast) and one layer comprised of both fetal and maternal cells (fetal trophoblast giant cells and maternal decidua). Along with simple changes in overall size, some hybrids are reported to show underlying changes in the cellular composition of the placenta, specifically in the labyrinthine trophoblast (Duselis and Vrana 2010; Zechner et al. 1996; Allen et al. 1993). To evaluate the potential for this bias in our study, we measured the relative area of each of the three placental cell layers (Table 1) and found no significant differences in the log-transformed relative area of the labyrinthine trophoblast between the two species and the reciprocal hybrids ( $F_{3,20} = 1.998$ ,  $P = 0.1467$ ). Thus, differences in gross histology are unlikely to strongly confound overall patterns of expression in our study. More subtle quantitative differences are possible but were not assessed here.

### *Disruption of placental expression in overgrown hybrids*

We sequenced late-term placental transcriptomes from 40 individuals (five males and five females from each of four cross types) with Illumina HiSeq 2000 paired-end 100bp sequencing resulting in an average of 106,291,974 read pairs per cross-type (10,629,197

read pairs per individual). This sequencing depth and biological replication is within recommended best practices guidelines (Auer and Doerge 2010; Todd, Black, and Gemmell 2016). We then constructed placental transcriptomes for each species using the *de novo* transcriptome assembly software *Trinity* (Grabherr et al. 2011). Using three-way reciprocal best BLAST searches between each species transcriptome and the mouse genome, we identified 12,843 genes including one mitochondrial, one Y-linked, 406 X-linked, and 12,435 autosomal.

To mitigate reference bias, we built species-specific pseudotranscriptomes using the *modtools* software suite (Huang et al. 2014; Huang et al. 2013; Holt et al. 2013). These pseudotranscriptomes maintained a common coordinate system while incorporating species-specific single nucleotide variants (SNVs) and insertion-deletion variation. All reads were mapped to both species-specific pseudotranscriptomes, merged based on mapping quality, and evaluated for differential expression with EdgeR (Robinson, McCarthy, and Smyth 2010). 8,381 autosomal genes (67%; FDR < 0.05) were differentially expressed (DE) in at least one pairwise comparison between the four cross types (Fig 1A). Overall, expression profiles grouped by cross type, with cross types further clustering with maternal environment, suggesting a maternal effect on the expression profiles in the placenta. Many of these differences (5,218 DE genes) reflected differential expression between *P. campbelli* and *P. sungorus*.

Species-specific expression differences were randomly distributed with respect to mouse chromosomal location (S1 Fig, FDR-corrected  $P > 0.05$ , hypergeometric test) and dominated by relatively moderate changes in expression levels. For example, only 1,444 DE genes showed at least one log fold change in expression level between the species. This

subset was enriched for genes associated with the innate immune system, carbohydrate binding, cognition, cell signaling, and enzyme inhibitor activity (S1 Table A), suggesting that the regulation of these pathways may be rapidly evolving in the hamster placenta. Similarly, many genes (4,290 autosomal, 199 X-linked) were differentially expressed between the reciprocal hybrids but only a small proportion of these show log fold changes greater than one (927 autosomal, 56 X-linked). These genes are enriched for genes involved in the innate immune system, hormonal regulation, channel activity, nucleotide receptor activity, peptidase activity, and ion channel activity (S1 Table B-D).

Next we focused on the 1,604 genes that showed transgressive expression in at least one hybrid type relative to both species (i.e., hybrid expression outside the range of both species). The vast majority of these transgressive differences were restricted to large hybrids (Fig 1B); 1,471 genes (1,398 autosomal, 73 X-linked) were significantly different between S×C and both species compared to just 204 (189 autosomal, 15 X-linked) transgressive genes in normal-sized c×s hybrids (Fig 1B,  $X^2_1 = 958.4$ ,  $P < 0.0001$ ). Only 71 genes with transgressive expression were shared between both reciprocal hybrids and this overlapping set was not enriched for any gene ontology enrichment categories. Given the same autosomal genotypes in reciprocal hybrids, this strong asymmetry indicates that most transgressive expression in our experiment was associated with abnormal hybrid growth and not interspecific hybridization *per se*.

We detected similar numbers of up- and down-regulated genes in S×C hybrids, but the directions of transgressive expression levels were strongly biased with respect to gene functions. Genes that were more highly expressed in S×C hybrids (678 genes) were enriched for mitotic and immune functions (Fig 1C, S1 Table E), while the 794 down-

regulated genes were enriched for general developmental processes such as angiogenesis, bone development, hormone receptor activity, and tissue morphogenesis (Fig 1D, S1 Table F). Functional asymmetry was much less apparent in c×s hybrids; 87 genes showed higher expression and were enriched for genes involved in the innate immune system (S1 Table G) while we detected no functional enrichment among the 117 genes with lower expression in c×s hybrids.

We next evaluated expression of the X chromosome. Female rodents are known to imprint the paternal X chromosome in the placenta (Dupont and Gribnau 2013; Latham 1996; Wake, Takagi, and Sasaki 1976) resulting in expression of only the maternal X chromosome. Comparisons between reciprocal hybrids or between a hybrid and its paternal species may therefore confound regulatory evolution of the X chromosome with disrupted expression. Therefore, to test for differential expression on the X chromosome, we compared each hybrid with its maternal species. In c×s hybrids, 25 X-linked genes were differentially expressed compared to *P. campbelli* versus 111 X-linked genes in S×C hybrids compared to *P. sungorus* ( $P < 0.0001$ , FET). This apparent asymmetry towards differential X-linked expression in S×C hybrids was similar to patterns of differential expression on the autosomes (Fig 1B) and the X chromosome was not enriched for differential expression (S1 Fig, FDR-corrected  $P > 0.05$ , hypergeometric test).

#### *ASE and genomic imprinting in dwarf hamsters*

RNA-seq data allow for the quantification of allele-specific expression in hybrid F1 genotypes by comparing the number of reads deriving from the maternal and paternal chromosomes (Xu Wang and Clark 2014). With reciprocal crosses, this general approach

can be used to test for parent-of-origin expression characteristic of genomic imprinting (DeVeale, van der Kooy, and Babak 2012; Xu Wang and Clark 2014; Wei and Wang 2013). We were able to quantify ASE for ~70% (9,043 of 12,843 genes) of the placental expressed genes in our dataset based on at least one diagnostic SNV between *P. campbelli* and *P. sungorus*.

We identified 88 autosomal genes with significant parent-of-origin ASE in *Phodopus* hamsters (Fig 2A), including 9 genes with predominantly paternal expression (maternally imprinted) and 79 genes with predominantly maternal expression (paternally imprinted). The X chromosome is paternally imprinted in the placenta of female rodents (Wake, Takagi, and Sasaki 1976; Latham 1996) and so to verify our ability to identify imprinted genes using patterns of ASE and test for the possible disruption of imprinted X chromosome inactivation (iXCI), we assayed the allelic expression of X-linked genes in females. 154 of the 156 variant-containing X-linked genes showed significantly biased maternal expression in females (Fig 2B). The two genes that showed appreciable paternal expression were *Kdm5c* and *Pola1*, both of which are known to escape iXCI in house mice (Berletch et al. 2015; Horvath, Li, and Carrel 2013; Wutz 2011; Yang et al. 2010; Nadaf et al. 2012). Unfortunately, there were no SNVs in *Xist* and so its expected paternal expression (Gayen et al. 2016; Kay et al. 1993) could not be evaluated. As a further verification of our approach, we found that the mitochondrial gene, *mt-Rnr2* exhibits maternal expression.

Imprinted genes often cluster across relatively broad chromosomal regions (millions of base pairs) in mammalian genomes. Though we do not currently have a physical genomic map for *Phodopus*, we found that 37 of the putative imprinted genes that we identified occurred in 8 clusters of at least 4 genes within 18 Megabases (Mb) bases of



each other in the mouse genome. This included two clusters that appear homologous to known imprinted regions in mice (S2 Table). To test whether this is more clustered than expected by chance, we generated 100,000 random bootstrap replicates of 88 genes drawn randomly without replacement from our placental transcriptome. The degree of chromosomal clustering in our ASE gene set fell within the top 10% of this distribution (S2 Fig). This pattern suggests some trend towards physical clustering, though this analysis is likely underpowered given the likelihood of considerable structural evolution between *Mus* and *Phodopus*.

The 88 autosomal genes that we identified with significant parent-of-origin ASE is comparable to the number of imprinted genes in *Mus* (149) (Morison, Ramsay, and Spencer 2005), *Homo* (81) (Pollard et al. 2007), and *Equus* (93) (Xu Wang et al. 2013). Eight imprinted genes overlapped between all four taxa where imprinting has been characterized on a genome-wide scale (Fig 3). Seventeen imprinted genes overlapped between *Phodopus* and *Mus*, which include well-known examples of genes expressed both paternally (*Dlk1*, *Igf2*, *Impact*, *Mest*, *Ndn*, *Peg3*, *Plagl1*, *Sgce*, *Snrpn*) and maternally (*Axl*, *H19*, *Osbpl5*, *Phlda2*, *Slc22a18*, *Tfpi2*, *Wt1*, *Zim1*) (Morison, Ramsay, and Spencer 2005). Thus, there is notably little overlap in putative imprinted gene sets in these diverse mammalian systems. These gene sets reflect diverse forms of data collected from a range of tissues, which likely account for some of the discrepancy. Variable ascertainment issues aside, these data suggest that genomic imprinting status is labile and rapidly evolving, and/or that strong parent-of-origin ASE also reflects other biological phenomena, or that our ASE analyses were prone to high false positive rates. As only the first two possibilities are biologically interesting, we conducted a detailed assessment of the factors known to

cause biases in ASE (DeVeale, van der Kooy, and Babak 2012; Xu Wang and Clark 2014; Proudhon and Bourc'h's 2010). Below we provide an overview of these analyses with additional details available as Supplemental Information.

Both analytical (e.g., reference mapping) and experimental (e.g., maternal contamination, library preparation) biases can result in spurious ASE patterns (DeVeale, van der Kooy, and Babak 2012; Xu Wang and Clark 2014; Proudhon and Bourc'h's 2010) and artificially high estimates of imprinting (Gregg, Zhang, Weissbourd, et al. 2010; Gregg, Zhang, Butler, et al. 2010). Through use of the pseudotranscriptomes, our bioinformatics analyses directly incorporated and mitigated issues associated with reference bias. The invasive nature of placentation inevitably results in some contribution of maternal tissue, which can lead to over-estimations of the number of maternally expressed genes (Xu Wang, Soloway, and Clark 2011; DeVeale, van der Kooy, and Babak 2012). Our data suggest a very strong enrichment of maternally expressed genes in hamster placenta. If maternal tissue contamination is driving this pattern, then we should see a transcriptome-wide bias towards maternal alleles. We modeled the frequency of allelic expression (P1 and P2) for all autosomal genes as a betabinomial distribution (Xu Wang and Clark 2014) and found that expression from the maternal allele was only slightly skewed from 50% in c×s samples ( $50.98 \pm 0.30\%$  maternal; t-test;  $P < 0.001$ ) and unbiased in S×C samples ( $50.04 \pm 0.13\%$  maternal; t-test;  $P = 0.322$ ). These results are similar to other studies where maternal contamination has been shown to be low (Xu Wang, Soloway, and Clark 2011) or absent (Xu Wang et al. 2013), suggesting that the strong enrichment of maternally expressed genes is not caused by widespread maternal contamination.

Another important consideration when estimating ASE from RNA-seq data is the

distribution of library complexity (Xu Wang and Clark 2014). Complexity bottlenecks during preparation can cause allelic skews and random dropout. Libraries with low complexity tend to have higher variance in allelic skew, resulting in many genes which falsely appear to show ASE (Xu Wang and Clark 2014). Following Wang and Clark (2014), we modeled the allelic distribution of each F1 hybrid library using a beta-binomial distribution and the over-dispersion parameter  $\rho$  (rho). Our libraries ranged from high to medium complexity ( $S \times C$   $\rho = 0.031-0.039$ ;  $c \times s$   $\rho = 0.043-0.068$ ; S3 Fig, see S1 Text for further discussion), falling within the range sufficient for estimating imprinted expression (Xu Wang and Clark 2014).

#### *Disruption of ASE in overgrown hybrids*

To evaluate the LOI model, we tested for asymmetry in the pattern of ASE between reciprocal F1 hybrids. Symmetric ASE is consistent with imprinted expression in both reciprocal hybrids. The LOI model applied to our system predicts asymmetric ASE in hybrids, specifically phenotypically normal hybrids ( $c \times s$ ) should show imprinted (mono-allelic or highly-biased) expression and the overgrown hybrids ( $S \times C$ ) should exhibit more bi-allelic expression concomitant with abnormal growth. We defined asymmetric ASE as genes with significant species-of-origin differences in expressed alleles between the reciprocal hybrids. Forty of the 88 autosomal ASE genes (45%) showed asymmetric ASE (Fig 2). No X-linked genes were found to have asymmetric ASE, indicating that that iXCI is maintained in both hybrids.

We first tested if genes with significant ASE were more likely than chance to show differential expression between the reciprocal hybrids. They were: 78% of the significant

ASE genes were differentially expressed between S×C and c×s compared to only 37% of autosomal genes (FET,  $P < 0.0001$ ), suggesting that imprinted genes are enriched for regulatory disruption in hybrids. We next tested four specific predictions of the LOI model: (1) asymmetric ASE should be associated with changes in the expression level of the gene. It is: genes with asymmetric ASE had on average a much greater difference in expression between S×C and c×s hybrids than did genes showing symmetric ASE (Fig 4,  $P < 0.0001$ , t-test). (2) Asymmetric ASE should primarily reflect a shift towards bi-allelic expression due to LOI in overgrown S×C hybrids placentas. It does: 39 of the 40 genes with asymmetric ASE showed bi-allelic expression (or less skewed ASE) in S×C hybrids (Fig 4B,  $X^2_1 = 36.1$ ,  $P < 0.0001$ ). (3) Asymmetric ASE in S×C hybrids should be functionally enriched for paternally expressed genes that tend to promote growth. Surprisingly, it does not. Only one paternally expressed gene showed asymmetric ASE in S×C hybrids versus 36 maternally expressed genes (Fig 4B). (4) Asymmetric ASE should correlate with an increased expression level in S×C hybrids showing bi-allelic expression. It does not: we found that 38 of the 39 genes with asymmetric ASE in S×C decrease in expression level concomitant with a gain of (paternal) expression (Fig 4B). Thus, the expression level and predicted functional roles of maternally- and paternally- expressed genes in our data are consistent with phenotypic patterns of hybrid placental overgrowth (*i.e.*, overgrown S×C hybrids show reduced expression of genes associated repression of growth), but were contrary to the predictions of the LOI model (*i.e.*, activation of an allele correlated with reduced, not increased overall expression).

#### *DNA methylation at candidate ASE genes*

Genomic imprinting is controlled through three major epigenetic mechanisms including allele-specific DNA methylation, histone modifications, and the expression of long non-coding antisense RNA (Ideraabdullah, Vigneau, and Bartolomei 2008; Bird and Wolffe 1999). The specific regulatory mechanisms controlling ASE remains unknown for many imprinted genes, even in the well-established mouse model system (Ideraabdullah, Vigneau, and Bartolomei 2008), and a detailed inquiry of these processes in dwarf hamsters is beyond the scope of the current study. The speciation literature has largely focused on LOI and increased expression through hypomethylation at CpG sites within promoter regions of imprinted genes (Vrana 2007; Wiley et al. 2008; Shi et al. 2005; Schütt et al. 2003; Brown, Piccuillo, and O'Neill 2012); a pattern that is largely not supported by our data (Fig 4). An alternative hypothesis is that the loss of ASE combined with reduced expression reflects silencing of both alleles through DNA hypermethylation.

We explored the association between patterns of ASE and DNA methylation. Using pyrosequencing of bisulfite-treated genomic DNA, we quantified patterns of DNA methylation at CpG sites within candidate promoter regions of one control gene (*H19*) with the same ASE pattern and expression level in reciprocal hybrids and two genes that show asymmetric ASE coincident with dramatically reduced expression level in S×C hybrids, (*Tfpi2* and *Wt1*). For each of these genes we identified CpG islands within putative promoter regions and assayed patterns of CpG methylation in a male and female from each parental species and each hybrid type (Fig 5). For *H19* we found that approximately 50% of chromosomes showed methylation in each species and the reciprocal hybrids as expected for an imprinted gene (Fig 5A). Though expected for this well-established locus, these data verify that patterns of ASE can be used to reliably identify imprinted loci in *Phodopus*.

Within the candidate promoter region of *Tfpi2* we found one CpG site 1,088 bp upstream of the transcription start site that showed ~50% methylation in all individuals consistent with allele-specific methylation and four other CpG sites that were hypomethylated in all genotypes. (Fig 5B). The assayed CpG island in *Wt1* fell within the first exon and showed no evidence of allele-specific methylation, nor correlations between ASE, expression level, and methylation status (Fig 5C). Thus, we found no evidence for change in methylation status (hyper- or hypo- methylation) in S×C hybrids relative to other cross types at any of the assayed CpG sites within *Tfpi2* or *Wt1*.

## Discussion

Disruption in gene expression has long been hypothesized to play a role in the evolution of reproductive isolation (Lifschytz and Lindsley 1972; Butlin et al. 2012; Ortíz-Barrientos, Counterman, and Noor 2006). Recent work has begun link the evolution of hybrid male sterility to the disruption of key regulatory processes on the X chromosome (Good et al. 2010; Turner et al. 2014), and multiple examples of specific hybrid incompatibilities appear to involve deleterious changes in aspects of gene expression (Scarpino et al. 2013; Michalak 2003; Haerty and Singh 2006; Renaut and Bernatchez 2010; Barreto, Pereira, and Burton 2015). However, it remains unclear if common forms of intrinsic hybrid sterility and inviability often reflect a common genetic basis that is regulatory in nature. Abnormal hybrid growth in mammals is broadly interesting in this context because it presents a recurrent and rapidly evolving form of hybrid inviability (Brekke and Good 2014) for which there are strong *a priori* reasons to suspect a regulatory basis involving genomic imprinting (Vrana 2007). In our study we found strong support for a link between disrupted gene expression, genomic imprinting, and extreme hybrid overgrowth in dwarf hamsters. Below we discuss the insights afforded by our data into the evolution of genomic imprinting, the relationship between gene expression and hybrid inviability, and the epigenetic basis of disrupted expression in hybrids.

### *Genomic imprinting in dwarf hamsters and other mammals*

We identified 88 genes with consistent parent-of-origin dependent ASE in dwarf hamsters, adding to a relatively small number of mammal systems where genome-wide patterns of

ASE have been used to infer patterns of genomic imprinting (Fig 3). Our candidates include many genes that are imprinted in other species as well as several that appear uniquely imprinted in hamsters. While the discovery of a large number of novel imprinted genes in hamsters should be treated with caution pending further verification, relatively few genes are shared among the other three species for which genome-wide ASE data are available (Fig 3) suggesting that imprinting status is relatively rapidly evolving in mammals.

Like the vast majority of imprinting or ASE studies (Crowley et al. 2015; Vrana et al. 1998; Xu Wang et al. 2013), our experiment leveraged crosses between divergent lineages. This approach is clearly limiting given that disruption of imprinting status appears to be a recurrent consequence of evolutionary divergence. The reasons for this common bias are simple. Estimation of ASE from RNA-seq requires genetic variation and studies between closely related genotypes are therefore strongly underpowered, especially in mammalian populations that tend to have relatively low levels of genetic diversity. Indeed, even in our interspecific crosses we were only able to estimate ASE for ~70% of expressed genes. The consequences of this bias for detecting and understanding the evolution of imprinting are less clear. For example, if LOI occurs frequently in hybrids but is not asymmetric in many cases, then we might expect little overlap in imprinted gene sets in comparisons between distantly related species.

The ASE genes that we have identified were strongly biased towards maternal expression and similar in many ways to early estimates from house mice (Morison, Ramsay, and Spencer 2005; Xu Wang et al. 2008). More recent work suggests a more equal number of maternally and paternally expressed genes in mouse placenta (Xu Wang, Soloway, and Clark 2011) or even a strong excess of paternally expressed genes in the



equine placenta (Xu Wang et al. 2013). These previous studies have argued that the earlier trend in mice towards maternally expressed genes was due to maternal contamination during tissue collection. While some inclusion of maternal tissue in whole placenta dissections is hard to avoid, we found little evidence that extensive maternal contamination influences genome-wide patterns of ASE in our study (Fig 2A). Rather maternal and paternal allelic proportions closely followed a 1:1 null expectation for the vast majority of genes. If the observed bias towards maternal expression does reflect tissue contamination, then this effect must also be restricted to relatively few genes overall in our study. The observation that genes with biased ASE expression in hamsters also tend to be spatially clustered within the mouse genome (S2 Fig) suggests that ASE at many of these genes reflects shared regulatory phenomena.

We still have no clear sense of what the relative frequency of maternal versus paternal imprinting should be in mammals or how this pattern might change as a function of evolutionary (e.g., intensity of paternal conflict) or physiological considerations (e.g., diversity in placental morphology). Nonetheless, the demonstration of a strong paternal bias in cultured equine placentas (where maternal contamination is impossible) suggests that imprinting in some species may be highly biased towards the expression of one parental type (Xu Wang et al. 2013). While there are almost certainly false-positives in the candidate imprinted genes we have identified, we believe that the overarching signal of our data robustly illustrates the relationship between allelic usage, expression level, and functional category of genes expressed in the placenta and provides fundamental insights into the relationship between gene expression and reproductive isolation (see below).

It is also important to acknowledge that maternal expression from the uterus or

decidua (the maternal component of the placenta) is likely to have important biological influences on offspring growth and should be taken into account when considering the functional consequences of hybridization. Maternal effects such as the uterine environment have been shown to greatly influence growth of horse hybrids (Allen et al. 2004; Allen, Wilsher, Turnbull, et al. 2002; Allen, Wilsher, Stewart, et al. 2002) and there appears to be a clear maternal effect in our data where the overall expression profiles of hybrids cluster most closely with the maternal species (Fig 1A). Though we see no signal of genome-wide maternal contamination, it is possible that interactions with maternal genes expressed in the uterus play an important role in the incompatibilities that underlie the disruptions of placental gene expression. Indeed, mammalian development requires the successful interaction between the mother and her offspring, as well as the proper interaction between maternally- and paternally- derived alleles within the offspring. Studying placental expression outside of the context of the maternal-fetal interaction may obscure such incompatibilities.

#### *Genomic imprinting, LOI, and abnormal hybrid growth*

Genomic imprinting may be involved in parent-of-origin placental growth of mammal hybrids for two *a priori* reasons. First, monoallelic expression of imprinted genes and (or) the X chromosome (in males or through iXCI in females (Wake, Takagi, and Sasaki 1976)) may expose deleterious recessive interactions in the placenta. Approximately half of the autosomal imprinted genes and nearly all X-linked genes are consistently imprinted in hybrid dwarf hamsters. While we have shown that autosomal genomic imprinting status appears highly susceptible to regulatory disturbance in overgrown hybrids, this is not true

for the imprinting of the X chromosome in female placentas. Proper iXCI was also found in house mouse hybrids (Hemberger et al. 2001) where an X-linked factor is also involved in parent-of-origin hybrid growth (Kropáčková et al. 2015; Zechner et al. 2004). Likewise, an interaction involving an X-linked gene thought to be *Esx1* and the imprinting status of *Peg3* is strongly associated with parent-of-origin growth in hybrid deer mice (Vrana et al. 2000; Loschiavo et al. 2007). If the X chromosome is also involved in parent-of-origin growth in hamsters, it is likely through similar negative epistatic interactions that in turn disrupt autosomal gene expression rather than through disruption of iXCI. Furthermore, the current experiments have not ruled out the involvement of mitochondrial-nuclear interactions contributing to the extreme overgrowth of S×C hybrid hamsters.

Second, disruptions in imprinting status may give rise to dosage imbalances of growth regulating genes. The LOI model has emerged as the predominant mechanistic basis for disrupted imprinting in hybrids (Vrana 2007; Crespi and Nosil 2013) and predicts that loss of imprinting should lead to increased overall expression due to the activation of a normally silenced allele. Although we found a strong association between ASE and overall expression levels in hybrids (Fig 4), the transition towards more equal usage of parental alleles was strongly negatively associated with expression levels at most genes. Several genes that were maternally expressed in normal c×s hybrids showed both bi-allelic expression (i.e., gain of paternal expression) coupled with a dramatic reduction in overall expression level in overgrown S×C hybrids. Importantly, this striking pattern is conservative to the potential issue of increased maternal contamination due, for example, to disrupted maternal-fetal interactions in overgrown hybrid placentas.

Changes in expression levels and ASE status at specific functional sets of genes were

all strongly consistent with the observed patterns of abnormal hybrid growth. Hybrid placental overgrowth in hamsters is strongly coupled with down-regulation of maternally expressed genes generally associated with negatively regulating offspring growth. These and other widespread changes in expression in overgrown S×C hybrids (Fig 1) are likely a consequence of deleterious Dobzhansky-Muller type interactions (Muller 1942; Dobzhansky 1936) between a much smaller set of loci. Thus, while the genetic and epigenetic bases of disrupted hybrid placental expression remain in question, our data support the general conclusion that changes in gene expression play an important causative role in the manifestation of hybrid inviability in dwarf hamsters.

Although the LOI model itself is not supported by the bulk of our data, several examples exist where gain in allelic expression is coupled with increased expression in hybrids (Vrana 2007; Crespi and Nosil 2013). At least some of these cases are likely to be explained by hypomethylation of the normally silenced allele, resulting in increased overall expression. This LOI model could explain the expression pattern of one gene in our data set: *Mest* (i.e., *Peg1*), though it should be noted that expression of *Mest* from the maternal tissue is a possible explanation as well. *Mest* is a paternally expressed growth promoter associated with abnormal placental phenotypes in *Mus* (Nishita et al. 1996). Similar to hamster hybrids, *Mest* shows LOI in large F1 *Mus* hybrids (Shi et al. 2005). In mice, LOI is coincident with hypomethylation (Shi et al. 2005) as predicted by this model. Other examples of genes where LOI and increased expression are coincident with hypomethylation include *Peg3* and *Snrpn* in large *Mus* hybrids (Shi et al. 2005), *Peg3* in large *Peromyscus* hybrids (Wiley et al. 2008), and three genes (*Plagl1*, *Snrpn*, and *Nnat*) in large cattle (*Bos*) hybrids (Z. Chen et al. 2015).

Intriguingly, the dosage of imprinted genes is proving to be an important factor in reproductive isolation in flowering plants as well as mammals (Gutierrez-Marcos et al. 2003). There is evidence that seed inviability is caused by genes with parent-of-origin effect including imprinted genes, in both *Mimulus* (Garner et al. 2016) and *Arabidopsis* (Wolff et al. 2015). Similar to the placenta of mammals, LOI and abnormal dosage of imprinted genes has been found in the endosperm of *Arabidopsis* hybrids (Josefsson, Dilkes, and Comai 2006; Jullien and Berger 2010). DNA methylation is also involved in regulating imprinting in angiosperms (Gehring 2013; Rodrigues and Zilberman 2015). Thus, abnormal methylation of imprinted genes in the endosperm presents an exciting parallel with the regulation of imprinting in the mammalian placenta and suggests a fundamental role of parent-offspring conflict as an ultimate driver of reproductive isolation in these disparate taxa.

These and other examples demonstrate that disruption of expression through LOI likely contributes to the evolution of growth-related hybrid incompatibilities in mammals and plants. However, patterns of ASE in hamsters, coupled with a reconsideration of broader trends in many other hybrid systems, suggest that the central importance of LOI needs to be reconsidered.

#### *Alternative mechanisms underlying patterns of disrupted ASE*

In the context of LOI, it is difficult to reconcile the apparent activation of an allele with the striking reduction in overall expression levels. This apparent contradiction may be resolved with a distinction between the gain of expression of an allele due to a loss of silencing (the standard LOI model) and a change in patterns of ASE due to other regulatory

mechanisms that impact maternal and paternal alleles equally. DNA methylation is often inversely correlated with the expression level of a gene (Newell-Price, Clark, and King 2000; Bird and Wolffe 1999; J. R. Mann et al. 2000), but in many instances the repressive epigenetic marks underlying imprinting do not result in complete silencing (see Fig 2A and Figure 2f in (Xu Wang and Clark 2014)). An alternative mechanism that could explain bi-allelic expression coupled with reduced expression level would be hypermethylation of both alleles resulting in increased but incomplete gene silencing (Bird and Wolffe 1999). Though not widely emphasized in the speciation literature, this unbiased silencing model could explain the unexpected low expression of many candidate LOI genes in hybrid cattle including the maternally expressed genes *Tfpi2*, and *Osbp15* (Z. Chen et al. 2015) and the maternally expressed *Dcn* and *Cd81* in deer mice (Wiley et al. 2008).

Both the loss of imprinting due to hypomethylation and the gain of imprinting due to hypermethylation are found in many instances of abnormal growth phenotypes associated with human diseases. For instance, Beckwith-Wiedmann syndrome is a human disease characterized by growth dysgenesis. Approximately 50% of cases are caused by LOI and hypomethylation of the long noncoding RNA *Kcnq1ot1*, while ~10% of cases are due to hypermethylation at the imprinted cluster containing *Igf2* and *H19* (Lim:2010fh Turan et al. 2010; Weksberg, Shuman, and Beckwith 2010; Horsthemke and Buiting 2008). Other examples of growth-related pathologies include Wilms tumors caused by hypermethylation of *Igf2/H19* promoter silencing *H19* expression (Bjornsson et al. 2007), Silver-Russell syndrome caused by hypomethylation of the *Igf2/H19* control region (Eggermann et al. 2015), and hydatiform moles that exhibit hypermethylation of genes normally paternally imprinted and hypomethylation at genes normally maternally imprinted (Sanchez-Delgado

et al. 2015; Judson et al. 2002).

Our initial attempt to test this hypermethylation model at two genes was uninformative (Fig 5) due to a lack of knowledge of how imprinting is regulated at these loci (and more generally in dwarf hamsters). *Tfpi2* and *Wt1* are both growth repressing genes that show drastically reduced expression along with bi-allelic expression in large hybrids. While there have been reports that CpG hypermethylation lowers *Tfpi2* expression in lung cancers (Rollin et al. 2005) and gastric cancer (Takada et al. 2010), there are also reports that imprinting of *Tfpi2* in the placenta may actually be regulated by histone methylation rather than DNA methylation (Monk et al. 2008). Similarly, *Wt1* imprinting has been reported to be regulated by CpG methylation and that hypermethylation of the promoter is known to occur (Hiltunen, Koistinaho, and Alhonen 1997; Kaneuchi et al. 2005), but others report that the imprinting of *Wt1* is regulated by the anti-sense long-noncoding RNA, *Wt1-as* and that hypomethylation of this gene results in LOI of *Wt1* (Malik et al. 2000). With such unknowns even in the mouse model system, it is perhaps unsurprising that our tests in dwarf hamsters were uninformative. While it is unfortunate that the assays of CpG methylation for these two genes did not conclusively test this alternative mechanism, there is ample evidence that further study along these lines are well motivated in dwarf hamsters and other hybrid systems.

## Methods

### *Animals*

Outbred colonies of dwarf hamster (*P. campbelli* and *P. sungorus*) were established as described in (Brekke and Good 2014) from wild-derived stocks collected in 1981 and 1990 (Scribner and Wynne-Edwards 1994). All animals were housed in a 14:10 light:dark regimen in accordance with University of Montana Institutional Animal Care and Use Committee regulations (animal use protocol 039-13JGDBS-090413).

### *Experimental crosses and phenotypic analyses*

We used 38 experimental crosses within and between *P. campbelli* and *P. sungorus* to generate placentas for sectioning (n=32 placentas) and RNA extractions (n=40 placentas, 5 males and 5 females for each of four cross types). Only placentas associated with viable embryos were used for downstream applications. Embryos were developmentally staged to ensure all samples were in the final days of gestation corresponding to Theiler's Stages 24-27 (Theiler 1972) as previously reported (Brekke and Good 2014). Placentas were snap-frozen on dry ice for RNA extractions or fixed in 4% paraformaldehyde overnight for histological analyses. Fixed placenta were then embedded in paraffin, sectioned at 7 microns, stained with hematoxylin and eosin (HE), and photographed with a Leica DM1000 microscope. As most placentas were too large to fit in a single frame, multiple pictures were taken and stitched together using *ImageJ* (Preibisch, Saalfeld, and Tomancak 2009; Schneider, Rasband, and Eliceiri 2012). The area of the labyrinthine layer, the spongiotrophoblast, and the trophoblast giant cells were measured in *ImageJ* and the relative size of each of these tissue layers was calculated by dividing by the total area of the



placenta.

#### *DNA extraction and sex-typing*

Genomic DNA was extracted from frozen embryos with a Machery-Nagel Nucleospin Tissue DNA extraction kit (740952). Standard kit protocols were followed with the exception that 5µl RNase-A was added to the column and incubated for 15 minutes at room temperature.

Sex was determined using a PCR amplification of the Y-linked gene *Sry* using the forward primer: 5`-CCCAGCATGCAAAAYWCAGA-3` and the reverse primer: 5`-

RTCTCTGNGCCTCCTGGAAA-3`. Reaction conditions were 1x[94c for 2:00], 30x[94c for 0:15, 60c for 0:20, 72c for 1:20], 1x[72c for 4:00, 10c hold]. Hybrid sex was secondarily confirmed using a previously published assay (Brekke and Good 2014) that relies on sequencing an X-linked SNV found between *P. campbelli* and *P. sungorus*.

#### *RNA extraction and Illumina library preparation*

RNA was extracted from whole frozen placentas with an E.Z.N.A. Micro RNA Kit (Omega) and treated with DNase. Whole placentas were ground on liquid nitrogen to completely homogenize the tissue. All RNA samples were quality checked with an Agilent 2100 Bioanalyzer and had RNA integrity numbers (RIN) greater than 8. Individually barcoded RNA-seq libraries were prepared with Illumina's TruSeq kit and recommended strand-specific modifications (Sultan et al. 2012) starting from 2 ng of starting RNA for each library. To determine the number of cycles for PCR amplification, 1µL of each library was amplified on a Agilent Mx3000 qPCR machine using a DyNAmo Flash SYBR Green qPCR kit (Thermo Scientific) and with primers designed to pair with the Illumina adapters

(reamp\_P5: AATGATACGGCGACCACCGA; and reamp\_P7: CAAGCAGAAGACGGCATACGA; reaction conditions: 1x[95c for 10:00], 40x[95c for 1:20, 65c for 0:30, 72c for 0:30], 1x[95c for 1:00, 55c ramp up to 95c, 95c for 0:30]). Based on the qPCR amplification curves, 8 $\mu$ L of each library was PCR amplified for 12 cycles (instead of the suggested 15) to avoid over-amplification and minimize PCR duplicates. The amplified libraries were then pooled and sequenced on two lanes of Illumina HiSeq 2000 using 100 bp paired-end sequencing. Individual libraries were prepared and distributed across the two Illumina lanes to achieve a balanced sequencing effort across treatments (Auer and Doerge 2010).

#### *Transcriptome generation, SNV calling, and pseudotranscriptome generation*

Illumina adapters were trimmed from the raw reads with Cutadapt (v1.6) using the parameters -O 5 and -e 0.1 (Martin 2011). Reads were next cleaned based on quality with Trimmomatic (v0.3.2) using the parameters: LEADING:5, SLIDINGWINDOW:4:15, MINLEN:36, and HEADCROP:13 (Bolger, Lohse, and Usadel 2014). Reads from the ten individuals of each parental species were pooled and used to create two species-specific transcriptomes with the program Trinity (v2013\_08\_14) (Grabherr et al. 2011). The transcriptomes had over 135,000 purported genes, however there are only around 25,000 known genes in most mammal genomes. In order to remove the spurious “genes” from these transcriptomes we used a reciprocal-best-blast-hit approach and blasted each transcriptome to the other and to the *Mus musculus* genome GRCm38 cds and ncRNA databases. Genes included in the final transcriptome were three-way reciprocal best blast hits. The final transcriptome includes 12,843 genes. We did not analyze expression of splice-variants due to the difficulties of accurately calling variants in the absence of a

genome. The longest variant was used for each gene.

Reads from both parental species were mapped to the transcriptome and SNVs were called between *P. campbelli* and *P. sungorus* using the HaplotypeCaller from GATK (v3.1-1) and the parameter `-stand_call_conf 30` (Van der Auwera et al. 2013; McKenna et al. 2010; DePristo et al. 2011). The SNVs were then filtered with `vcftools` (v0.1.1) on number of alleles (`--min-alleles 2, --max-alleles 2`), and site quality (`--minQ 500`). Finally, sites were selected that were fixed for alternative alleles in each species. This resulted in 83,230 SNV calls spread across 9,043 genes.

We generated a pseudotranscriptome for each species using the `modtools` package (v1.0.5) (Huang et al. 2013; Holt et al. 2013). Pseudotranscriptomes use a common coordinate system while accounting for SNVs and insertion/deletions (indels) in order to both eliminate mapping bias and identify the parental origin of each mapped read in an F1 individual. By aligning the reads of an individual to both the maternal and paternal pseudotranscriptomes and comparing the mapping quality of each read, reads are assigned a parent of origin. In accordance with the `modtools` pipeline, reads from all individuals were mapped to both the *P. campbelli* and *P. sungorus* pseudotranscriptomes using Bowtie2 with the `--end-to-end` parameter and the coordinates were then converted to the reference coordinate system. Next the alignments from the maternal and paternal pseudotranscriptomes were merged and reads were assigned a parent of origin.

### *Gene location annotation*

In order to annotate genes as either autosomal, mitochondrial, X-, or Y-linked we used homology with *Mus musculus*. If a gene is X-linked, Y-linked or mitochondrial in the house

mouse, we assumed that it is similar in *Phodopus*. Then, to refine our predictions, we surveyed all hybrid males for heterozygous expression in putative X genes. As males only have a maternally inherited mitochondria and X chromosome, any putative mitochondrial or X gene with more than 1% expression of the paternal SNV across all 10 hybrid males was reclassified as autosomal. Similarly we affirmed that Y-linked genes showed no female expression. This resulted in 12,437 autosomal genes, 406 X-linked genes, one Y-linked gene (*Kdm5d*) and one mitochondrial gene (*mt-Rnr2*).

#### *Differential expression and allele-specific expression analyses*

To evaluate differential expression, we created a table of counts at the gene level incorporating all reads regardless of parental origin using `featureCounts` (v1.4.2) in order to count fragments (-p) and discard those that have too long an insert (-P) or are chimeric (-C) or have a mapping quality (-Q) below 20 (Liao, Smyth, and Shi 2014). Differential expression was evaluated from this table of counts with the generalized linear model approach implemented in `edgeR` (Robinson, McCarthy, and Smyth 2010; McCarthy, Chen, and Smyth 2012). Expression profiles were grouped by gene and individual with the function `hclust()` in *R*.

In order to assay allele-specific expression, reads originating from the maternal or paternal genomes were identified with the `modtools` pipeline (Huang et al. 2013). `featureCounts` was used to build a table of parent-specific counts including all of the 20 F1 individuals, using only reads that were definitively assigned a parental origin (*i.e.*, those spanning a SNV or indel), and using the same flags as above. Using this method, our evaluation of allelic expression incorporates expression across all variants in each gene and

is thus robust to discordance in allelic expression across linked SNVs (DeVeale, van der Kooy, and Babak 2012). Genes that show a negative correlation in the number of maternal vs paternal reads across individuals are assumed to be unreliable and so 606 out of 9,043 genes were excluded from analyses of allele-specific expression. While 606 is only a small fraction of the total number of genes, they comprised a large proportion of false-positive imprinting calls. All individuals were used to generate allele-specific significance estimates for autosomal genes, while only females were used for X-linked genes. For each gene with one or more diagnostic positions, we transformed the counts of allele-specific expression into Wang and Clark's P1 and P2 metric (Xu Wang and Clark 2014). Here we define P1 as the proportion of *P. campbelli* expression in c×s hybrids while P2 is the proportion of *P. campbelli* expression in S×C hybrids. We used the EdgeR framework to estimate differential expression between maternal reads and paternal reads across all individuals. Candidate imprinted genes were identified based on a significant skew in the parent-of-origin of expressed reads using an FDR corrected *P*-value of 0.05. We also required that potential imprinted genes must show, at minimum, a difference in allelic skew between the hybrids of 0.4 (*i.e.*,  $|P2-P1| > 0.4$ ) to define genes with significant maternally- ( $P2 < P1 - 0.4$ ) or paternally- ( $P2 > P1 + 0.4$ ) biased ASE. Though arbitrary, this cutoff should largely account for and exclude genes with slight but highly significant parent-of-origin allelic skews; a seemingly common pattern in transcriptome data that may reflect either expression biases thought to be distinct from traditional genomic imprinting (Crowley et al. 2015) or sufficiently high sequencing coverage that minute allele-specific differences become statistically significant. To confirm that our results are not dependent on an arbitrarily defined breakpoint, we repeated all analyses with cutoffs of 0.0 (no cut-off; any gene with

significant allelic skew is accepted), 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 (only genes expressed perfectly from one parental allele are included as “imprinted” - there were none of these). Though the absolute number of candidate imprinted genes is naturally dependent on the cutoff, our major findings concerning how expression level changes when imprinting is disrupted are robust across all cutoffs (S4 Fig).

To identify genes that show disrupted imprinting, we again used the EdgeR framework but this time tested whether genes showed significant species-bias in expression level. Genes with significant ASE are considered to show asymmetric ASE (i.e. ASE in one hybrid but not the other) if they show significant skew in the species identity of the alleles with an FDR-corrected  $P$ -value of 0.05 and if either P1 or P2 falls between 0.15 and 0.85. A list of all candidate imprinted genes can be found in S1 Table.

#### *Annotating imprinted genes into clusters*

We annotated imprinted genes into clusters based on the locations of their homologs in the *Mus musculus* genome (GRCm38). We identified sets of genes from the same chromosome that had less than 18MB between start sites. The groups with more than four members were defined as clusters. We repeated this analysis 100,000 times with groups of 88 genes randomly drawn without replacement to establish a null expectation for whether our putative imprinted genes are more- or less- clustered than expected based on chance.

#### *Methylation assays*

In order to assay the regulatory regions of three candidate genes for disrupted patterns of methylation we used the Pyro-Mark Q96 ID to sequence bisulfite-treated genomic DNA.

DNA was extracted from whole placenta using the Qiagen DNeasy Blood and Tissue Kit (Qiagen cat. no. 69504) after homogenization with liquid nitrogen. It was then treated with bisulfite with the EZDNA methylation kit (Zymo Research, cat. no. D5001). Primers (Left-, biotinylated Right-, and Sequencing-) specific for bisulfite treated DNA were designed with the Pyro-Mark assay design software (v2.0, Qiagen). *H19* primers are: H19\_L1\_Methyl: AGGATGAAGTAGGGTATGTTG, H19\_R1\_Methyl\_5'biotin: TATCACCCCAATACCTACCTATCATC, H19\_S1\_Methyl: GAATATTTTGATGGAATTGTTT and amplify a CpG island found near the beginning of first exon of *H19*. *Wt1* primers are: Wt1\_L1\_Methyl: GGGGGATAGAGGTTTTGTAGTTT, Wt1\_R1\_Methyl\_5'biotin: CCCCAACTAACTCCTATTAA, Wt1\_S1\_Methyl: GTAGTTTTTGGGTAAAGTTT, and amplify a CpG island in the first exon of *Wt1*. *Tfpi2* primers were designed to amplify two regions upstream of *Tfpi2* start site that contain CpG islands. These are: Tfpi2\_L1\_Methyl: GGGATGGGTATTGTTTTAGGTATG, Tfpi2\_R1\_Methyl\_5'biotin: AACCTATCACTACAACCTTTAAACAAAC, Tfpi2\_S1\_Methyl: TGTTTTTAGGTATGAATTAGTTATAT, Tfpi2\_L2\_Methyl: AGATGGAGGTAGGAGGATAAA, Tfpi2\_R2\_Methyl\_5'biotin: ACATTAAATCCAACAAAATAACACTCA, Tfpi2\_S2\_Methyl: TTTTGATTTGGGTGGT. All CpG islands were defined by the program CpGIslandFrame (v1.0). DNA was treated with bisulfite and amplified in duplicate with the following PCR protocol: 1x[95c for 15:00], 50x[94c for 0:30, 54c for 0:30, 72c for 0:30], 1x[72c for 10:00, 10c hold]. Pyrosequencing was completed as per the manufacturers directions and CpG methylation levels were evaluated with the PyroMark-CpG software. Non-CpG sites were used as an internal control for bisulfite DNA conversion and percent methylation was evaluated on a site-by-site basis.

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## Figure Legends

**Fig 1. Overgrown hybrids have an excess of differentially expressed autosomal genes.** (A) Clustering of 8,218 autosomal genes that show differential expression in any pairwise comparison between cross types. Columns represents individuals and rows represent genes, the color represents the scaled expression level. (B) Venn diagram of 1,604 genes showing transgressive expression levels (significantly higher or lower) in one or both hybrids relative to both expression species. Normalized expression and patterns of gene ontology enrichment for (C) 678 genes where S×C hybrids have higher expression than the parental species and (D) 794 genes where S×C hybrids have lower expression than the parental species. Individual genes are shown with gray lines and the group average is shown in red.

**Fig 2. Allele-specific expression.** The proportion of *P. campbelli* allelic expression for c×s hybrids (P1) plotted against the proportion of *P. campbelli* allelic expression in S×C hybrids (P2). Histograms display density along each axis. (A) Autosomal genes with paternally expressed genes lie in the upper left and maternally expressed genes lie in the lower right. Candidate imprinted genes at the  $|P1-P2|>0.4$  cutoff are colored yellow for maternal genes and blue for paternal genes. Genes that show significant differences in ASE between hybrids are circled with red. (B) All X-linked genes show maternal expression in females consistent with imprinted X-chromosome inactivation (iXCI) of the paternal X chromosome, with the exception of two genes (*Kdm5c* and *Pola1*) that partially escape iXCI.

**Fig 3. Mammalian imprinted genes.** Venn diagram of putative imprinted genes in four groups of placental mammals - humans (*Homo*), mice (*Mus*), horses (*Equus*), and hamsters (*Phodopus*) - where parent-of-origin expression has been assayed on a genome-wide level. The data are from the current study (*Phodopus*), the parent-of-origin effect database (*Homo* and *Mus*; (Morison, Ramsay, and Spencer 2005), and a previous study in *Equus* (Xu Wang et al. 2013). The eight genes common to all species are *Dlk1*, *Igf2*, *Mest*, *Ndn*, *Peg3*, *Sgce* (paternally expressed) and *H19*, and *Phlda2* (maternally expressed).

**Fig 4. Disrupted ASE is coupled with reduced expression.** Comparison of normalized expression levels between reciprocal hybrids for (A) 48 genes with symmetric patterns of ASE and (B) 40 genes with significant differences in ASE between hybrids. Paternally expressed genes are colored blue, maternally expressed genes are colored yellow. Log fold changes are polarized so that positive and negative values reflect higher or lower expression in S×C hybrids respectively, and (\*) indicates significant differential expression (FDR-corrected  $P < 0.05$ ). Genes with symmetric ASE in both hybrids showed little trend towards higher- or lower expression in overgrown S×C hybrids. Genes with asymmetric ASE (consistent with a change in imprinting status in one hybrid) were more common in S×C hybrids and showed a greater magnitude of expression change than symmetrically imprinted genes ( $P < 0.0001$ , t-test). Shifts towards bi-allelic expression in S×C hybrids was predominately detected in maternally expressed genes that also showed drastic reductions in overall expression levels.

**Fig 5. CpG methylation at three candidate imprinted genes.** Annotation of (A) *H19*, (B) *Tfpi2*, and (C) *Wt1* is predicted based on *Mus* (GRCm38). Horizontal lines represent non-exonic regions, narrow boxes represent untranslated exons, wide boxes represent coding domain sequence, and vertical marks below the sequence represent targeted CpG sites in dwarf hamsters numbered relative to the transcription start site. For each CpG site, the proportion of methylated (black) to unmethylated (white) sequences based on pyrosequencing of bisulfite-treated placental genomic DNA. Approximately 50% of chromosomes were methylated at *H19* in all genotypes, consistent with imprinting controlled by DNA methylation at these sites. A similar pattern was found at one CpG site in *Tfpi2* while other sites in *Tfpi2* and *Wt1* showed low levels of methylation suggesting that the imprinting of *Tfpi2* and *Wt1* is not controlled by methylation at these sites. No sites showed qualitative differences between cross types.

## Supporting Information

**S1 Fig. Chromosomal distribution of differentially expressed genes.** Patterns of differential expression between (A) *P. campbelli* and *P. sungorus* and (B) S×C hybrids versus the parental species. The line shows the 1:1 null expectation where the number of DE genes is purely a function of the number of genes on the chromosome. Any points below the line would represent chromosomes with more DE genes than expected by chance and imply a chromosome-wide regulatory disruption, however no chromosomes showed significant deviations from null expectations in either comparison (hypergeometric tests, FDR-corrected  $P < 0.05$ ). Expected and observed counts are based on the number of differentially expressed genes relative to all expressed genes and their chromosomal

location in the *Mus* genome. For comparisons involving S×C (*i.e.*, panel **B**), differential expression was evaluated between S×C and the parental mean for autosomal genes but between S×C and *P. sungorus* for X-linked genes as *P. sungorus* is the X-donating parent.

**S2 Fig. Physical clustering of imprinted genes identified using ASE.** Shown is the bootstrap distribution (100,000 replicates) of physical clustering within the mouse genome (GRCm38) for 88 genes sampled randomly from the *Phodopus* placental transcriptome. Physical clusters are defined as groups of at least 4 genes where the nearest neighbor of each gene in the set is no more than 18Mb away. The mean number of genes that cluster under these requirement out of a randomly drawn 88 is 26.7 with a standard deviation of 7.5. The vertical black line denotes the observed data of 37 of the genes with significant ASE fitting this criterion ( $P = 0.095$ ).

**S3Fig. Library complexity.** Library complexity was modeled as a betabinomial distribution defined by the overdispersion parameter  $\rho$ . **(A)** Overdispersion of ASE was significantly different between the two hybrid types (t-test,  $P < 0.0001$ ), suggesting a treatment effect or biological differences in the degree of ASE between the two cross-types. **(B)** Linear correlation of ASE between libraries within each cross-type based on on all 45 pairwise correlations in P1 from S×C libraries compared with all 45 pairwise correlations of P2 from S×C libraries (t-test,  $P < 0.0001$ ). If bottleneck events during library preparation drive low library complexity (high  $\rho$ ) in c×s libraries, then we would expect that the correlation in allelic expression across those libraries should be lower when compared to S×C libraries. The correlation in ASE among c×s individuals was actually significantly

higher. **(C)** If high  $\rho$  values are driven by the presence of imprinted genes and are thus due to biological differences between the reciprocal hybrids (rather than for instance, poor library preparation) then removing the small set of ASE genes and re-modeling  $\rho$  should result in the disappearance of the treatment effects. Consistent with this, reciprocal hybrids show similar levels of dispersion once the 88 ASE genes are removed.

**S4 Fig. Degree of allelic skew and imprinting.** To avoid over-estimating the number of imprinted genes, we followed the recommendations of Wang and Clark (Xu Wang and Clark 2014) and applied a hard cutoff for the ASE threshold. Only genes that are significantly different from 1:1 maternal:paternal and fall outside the cutoff threshold were considered potentially imprinted. We repeated our analysis across a broad range of possible thresholds to determine the effect of different thresholds on our results. **(A)** The number of genes with significant ASE was inversely related to the threshold. Unsurprisingly, more stringent thresholds exclude more genes and this is consistent between genes with both symmetric and asymmetric ASE. **(B)** The observation of asymmetric ASE (bi-allelic in one hybrid but not the other) with reduced expression level (rather than increased expression level as predicted by the LOI model) is robust across the entire range of thresholds.

## Tables

Table 1. Percent composition for each placental cell type.

Cross type	N	Labyrinthine Trophoblast	Sungio- trophoblast	Trophoblast Giant Cells
<i>P. campbelli</i>	6	62.3±3.0	18.2±1.9	19.5±1.5
<i>P. campbelli</i> × <i>P. sungorus</i>	8	66.7±3.8	17.7±2.2	15.0±1.6
<i>P. sungorus</i> × <i>P. campbelli</i>	5	54.9±3.9	24.6±1.6	20.3±3.1
<i>P. sungorus</i>	5	62.0±1.5	22.5±1.2	14.5±0.6

## Literature Cited

Abzhanov, Arhat, Meredith Protas, B Rosemary Grant, Peter R Grant, and Clifford J Tabin.

2004. "Bmp4 And Morphological Variation of Beaks in Darwin's Finches." *Science* 305 (5689): 1462–65. doi:10.1126/science.1098095.

Allen, W R, J A Skidmore, F Stewart, and D F Antczak. 1993. "Effects of Fetal Genotype and Uterine Environment on Placental Development in Equids." *Reproduction* 98 (1): 55–60. doi:10.1530/jrf.0.0980055.

Allen, W R, S Wilsher, C Turnbull, and F Stewart. 2002. "Influence of Maternal Size on Placental, Fetal and Postnatal Growth in the Horse. I. Development in Utero." *Reproduction* 123: 445–53.

Allen, W R, S Wilsher, F Stewart, J Ousey, and A Fowden. 2002. "The Influence of Maternal Size on Placental, Fetal and Postnatal Growth in the Horse. II. Endocrinology of Pregnancy." *Journal of Endocrinology* 172: 237–46.

Allen, W R, Sandra Wilsher, Clare Tiplady, and R M Butterfield. 2004. "The Influence of Maternal Size on Pre- and Postnatal Growth in the Horse: III Postnatal Growth" 127 (127): 67–77.

Auer, P L, and R W Doerge. 2010. "Statistical Design and Analysis of RNA Sequencing Data." *Genetics* 185 (2): 405–16. doi:10.1534/genetics.110.114983.

Barlow, Denise P. 1993. "Methylation and Imprinting: From Host Defense to Gene

- Regulation?" *Science* 260 (5106): 309–10.
- Barreto, F S, R J Pereira, and R S Burton. 2015. "Hybrid Dysfunction and Physiological Compensation in Gene Expression." *Molecular Biology and Evolution* 32 (3): 613–22. doi:10.1093/molbev/msu321.
- Berletch, Joel B, Wenxiu Ma, Fan Yang, Jay Shendure, William S Noble, Christine M Disteche, and Xinxian Deng. 2015. "Escape From X Inactivation Varies in Mouse Tissues." Edited by Marisa S Bartolomei. *PLoS Genetics* 11 (3): e1005079. doi:10.1371/journal.pgen.1005079.
- Bird, A P, and A P Wolffe. 1999. "Methylation-Induced Repression—Belts, Braces, and Chromatin." *Cell* 99 (5): 451–54. doi:10.1016/s0092-8674(00)81532-9.
- Bjornsson, Hans T, Lindsey J Brown, M Danielle Fallin, Michael A Rongione, Marina Bibikova, Eliza Wickham, Jian-Bing Fan, and Andrew P Feinberg. 2007. "Epigenetic Specificity of Loss of Imprinting of the *Igf2* Gene in Wilms Tumors." *Journal of the National Cancer Institute* 99 (16): 1270–73. doi:10.1093/jnci/djm069.
- Bolger, Anthony M, Marc Lohse, and Bjoern Usadel. 2014. "Trimmomatic: a Flexible Trimmer for Illumina Sequence Data." *Bioinformatics* 30 (15). Oxford University Press: 2114–20. doi:10.1093/bioinformatics/btu170.
- Brekke, Thomas D, and Jeffrey M Good. 2014. "Parent-of-Origin Growth Effects and the Evolution of Hybrid Inviability in Dwarf Hamsters." *Evolution* 68 (11): 3134–48. doi:10.1111/evo.12500.



- Brown, J D, V Piccuillo, and R J O'Neill. 2012. "Retroelement Demethylation Associated with Abnormal Placentation in *Mus Musculus* X *Mus Caroli* Hybrids." *Biology of Reproduction* 86 (3): 88–88. doi:10.1095/biolreprod.111.095273.
- Buiting, K, S Saitoh, S Gross, B Dittrich, and S Schwartz. 1995. "Inherited Microdeletions in the Angelman and Prader–Willi Syndromes Define an Imprinting Centre on Human Chromosome 15." *Nature* 377 (4): 395–400. doi:10.1038/ng0495-395.
- Butlin, Roger, Allan DeBelle, Claudius Kerth, Rhonda R Snook, Leo W Beukeboom, Ruth F Castillo Cajas, Wenwen Diao, et al. 2012. "What Do We Need to Know About Speciation?" *Trends in Ecology & Evolution* 27 (1): 27–39. doi:10.1016/j.tree.2011.09.002.
- Chakraborty, R. 1989. "Can Molecular Imprinting Explain Heterozygote Deficiency and Hybrid Vigor?" *Genetics* 122 (3): 713–17. doi:10.2307/2469503?ref=no-x-route:d07c394c5181b00fa6da1a3ec7754efa.
- Chen, Zhiyuan, Darren E Hagen, Christine G Elisk, Tieming Ji, Collin James Morris, Laura Emily Moon, and Rocío Melissa Rivera. 2015. "Characterization of Global Loss of Imprinting in Fetal Overgrowth Syndrome Induced by Assisted Reproduction." *Proceedings of the National Academy of Sciences* 112 (15): 4618–23. doi:10.1073/pnas.1422088112.
- Chen, Zhiyuan, Katherine Marie Robbins, Kevin Dale Wells, and Rocío Melissa Rivera. 2014. "Large Offspring Syndrome." *Epigenetics* 8 (6): 591–601. doi:10.4161/epi.24655.

- Cohn, M J, and C Tickle. 1999. "Developmental Basis of Limblessness and Axial Patterning in Snakes." *Nature* 399 (6735): 474–79. doi:10.1038/20944.
- Crespi, Bernard J, and Christina Semeniuk. 2004. "Parent-Offspring Conflict in the Evolution of Vertebrate Reproductive Mode." *The American Naturalist* 163 (5): 635–53. doi:10.1086/382734.
- Crespi, Bernard J, and Patrik Nosil. 2013. "Conflictual Speciation: Species Formation via Genomic Conflict." *Trends in Ecology & Evolution* 28 (1): 48–57. doi:10.1016/j.tree.2012.08.015.
- Crowley, James J, Vasyl Zhabotynsky, Wei Sun, Shunping Huang, Isa Kemal Pakatci, Yunjung Kim, Jeremy R Wang, et al. 2015. "Analyses of Allele-Specific Gene Expression in Highly Divergent Mouse Crosses Identifies Pervasive Allelic Imbalance." *Nature Genetics*, March. Nature Publishing Group, 1–10. doi:10.1038/ng.3222.
- DePristo, Mark A, Eric Banks, Ryan Poplin, Kiran V Garimella, Jared R Maguire, Christopher Hartl, Anthony A Philippakis, et al. 2011. "A Framework for Variation Discovery and Genotyping Using Next-Generation DNA Sequencing Data." *Nature Genetics* 43 (5): 491–98. doi:10.1038/ng.806.
- DeVeale, Brian, Derek van der Kooy, and Tomas Babak. 2012. "Critical Evaluation of Imprinted Gene Expression by RNA-Seq: a New Perspective." Edited by Anne C Ferguson-Smith. *PLoS Genetics* 8 (3): e1002600. doi:10.1371/journal.pgen.1002600.g006.

- Dobzhansky, T G. 1936. "Studies on Hybrid Sterility. II. Localization of Sterility Factors in *Drosophila Pseudoobscura* Hybrids." *Genetics* 21 (2):113.
- Dupont, Cathérine, and Joost Gribnau. 2013. "Different Flavors of X-Chromosome Inactivation in Mammals." *Current Opinion in Cell Biology* 25 (3): 314–21.  
doi:10.1016/j.ceb.2013.03.001.
- Duselis, Amanda R, and P B Vrana. 2007. "Assessment and Disease Comparisons of Hybrid Developmental Defects." *Human Molecular Genetics* 16 (7): 808–19.  
doi:10.1093/hmg/ddm025.
- Duselis, Amanda R, and Paul B Vrana. 2010. "Aberrant Growth and Pattern Formation in *Peromyscus* Hybrid Placental Development." *Biology of Reproduction* 83 (6): 988–96.  
doi:10.1095/biolreprod.110.085654.
- Duselis, Amanda R, Christopher D Wiley, Michael J O'Neill, and Paul B Vrana. 2005. "Genetic Evidence for a Maternal Effect Locus Controlling Genomic Imprinting and Growth." *Genesis* 43 (4): 155–65. doi:10.1002/gene.20166.
- Duselis, Amanda R, Craig Obergfell, Jennifer A Mack, Michael J O'Neill, Quang K Nguyen, Rachel J O'Neill, and Paul B Vrana. 2007. "Changes in Cell Cycle and Extracellular Matrix Gene Expression During Placental Development in Deer Mouse (*Peromyscus*) Hybrids." *Reproduction, Fertility and Development* 19 (5): 695.  
doi:10.1071/RD07015.
- Eggermann, T, N Schönherr, E Meyer, C Obermann, M Mavany, K Eggermann, M B Ranke,

- and H A Wollmann. 2006. "Epigenetic Mutations in 11p15 in Silver-Russell Syndrome Are Restricted to the Telomeric Imprinting Domain." *Journal of Medical Genetics* 43 (7): 615–16. doi:10.1136/jmg.2005.038687.
- Eggermann, Thomas, Guiomar Perez de Nanclares, Eamonn R Maher, I Karen Temple, Zeynep Tümer, David Monk, Deborah J G Mackay, et al. 2015. "Imprinting Disorders: a Group of Congenital Disorders with Overlapping Patterns of Molecular Changes Affecting Imprinted Loci." *Clinical Epigenetics* 7 (123). Clinical Epigenetics: 1–18. doi:10.1186/s13148-015-0143-8.
- Garner, Austin G, Amanda M Kenney, Lila Fishman, and Andrea L Sweigart. 2016. "Genetic Loci with Parent-of-Origin Effects Cause Hybrid Seed Lethality in Crosses Between *Mimulus* Species." *The New Phytologist*, February. doi:10.1111/nph.13897.
- Gayen, Srимonta, Emily Maclary, Michael Hinten, and Sundeep Kalantry. 2016. "Sex-Specific Silencing of X-Linked Genes by *Xist* RNA" 113 (3): E309–18. doi:10.1073/pnas.1515971113.
- Gehring, Mary. 2013. "Genomic Imprinting: Insights From Plants." *Annual Review of Genetics* 47 (1): 187–208. doi:10.1146/annurev-genet-110711-155527.
- Good, Jeffrey M, Thomas Giger, Matthew D Dean, and Michael W Nachman. 2010. "Widespread Over-Expression of the X Chromosome in Sterile F1 Hybrid Mice." *PLoS Genetics* 6 (9). Public Library of Science: e1001148. doi:10.1371/journal.pgen.1001148.

Grabherr, Manfred G, Brian J Haas, Moran Yassour, Joshua Z Levin, Dawn A Thompson, Ido

Amit, Xian Adiconis, et al. 2011. "Full-Length Transcriptome Assembly From RNA-

Seq Data Without a Reference Genome." *Nature Biotechnology* 29 (7): 644–52.

doi:10.1038/nbt.1883.

Gregg, C, J Zhang, B Weissbourd, S Luo, G P Schroth, D Haig, and C Dulac. 2010. "High-

Resolution Analysis of Parent-of-Origin Allelic Expression in the Mouse Brain."

*Science* 329 (5992): 643–48. doi:10.1126/science.1190830.

Gregg, C, J Zhang, J E Butler, D Haig, and C Dulac. 2010. "Sex-Specific Parent-of-Origin Allelic

Expression in the Mouse Brain." *Science* 329 (5992): 682–85.

doi:10.1126/science.1190831.

Gutierrez-Marcos, J F, P D Pennington, L M Costa, and H G Dickinson. 2003. "Imprinting in

the Endosperm: a Possible Role in Preventing Wide Hybridization." *Philosophical*

*Transactions of the Royal Society B: Biological Sciences* 358 (1434): 1105–11.

doi:10.1098/rstb.2003.1292.

Haerty, Wilfried, and Rama S Singh. 2006. "Gene Regulation Divergence Is a Major

Contributor to the Evolution of Dobzhansky-Muller Incompatibilities Between

Species of *Drosophila*." *Molecular Biology and Evolution* 23 (9): 1707–14.

doi:10.1093/molbev/msl033.

Haig, D. 1996. "Placental Hormones, Genomic Imprinting, and Maternal—Fetal

Communication." *Journal of Evolutionary Biology* 9 (3): 357–80.

- Haig, D. 2000. "The Kinship Theory of Genomic Imprinting." *Annual Review of Ecology, Evolution, and Systematics* 31: 9–32.
- Haig, David, and R Trivers. 1995. "The Evolution of Parental Imprinting: a Review of Hypotheses." In *Genomic Imprinting: Causes and Consequences*, edited by Rolf Ohlsson, K Hall, and M Ritzen, 1st ed., 17–29. Cambridge: Cambridge Univ Press.
- Hall, J G. 1990. "Genomic Imprinting: Review and Relevance to Human Diseases." *The American Journal of Human Genetics* 46: 857–73.
- Hemberger, M C, H Kurz, Annie Orth, Sabine Otto, A Lüttges, Rosemary W Elliott, Andras Nagy, et al. 2001. "Genetic and Developmental Analysis of X-Inactivation in Interspecific Hybrid Mice Suggests a Role for the Y Chromosome in Placental Dysplasia." *Genetics* 157 (1): 341–48.
- Hiltunen, M O, J Koistinaho, and L Alhonen. 1997. "Hypermethylation of the *Wt1* And Calcitonin Gene Promoter Regions at Chromosome 11p in Human Colorectal Cancer." *British Journal of Cancer* 76 (9): 1124–30. doi:10.1038/bjc.1997.522.
- Holt, J, S Huang, L McMillan, and W Wang. 2013. "Read Annotation Pipeline for High-Throughput Sequencing Data." *Acm-Bcb* 2013. doi:10.1145/2506583.2506645.
- Horsthemke, Bernhard, and Karin Buiting. 2008. "Genomic Imprinting and Imprinting Defects in Humans." *Advances in Genetics* 61: 225–46. doi:10.1016/S0065-2660(07)00008-9.
- Horvath, Lindsay M, Nan Li, and Laura Carrel. 2013. "Deletion of an X-Inactivation

- Boundary Disrupts Adjacent Gene Silencing.” Edited by Marisa S Bartolomei. *PLoS Genetics* 9 (11): e1003952. doi:10.1371/journal.pgen.1003952.
- Huang, Shunping, Chia-Yu Kao, Leonard McMillan, and Wei Wang. 2013. “Transforming Genomes Using MOD Files with Applications.” *Acm-Bcb 2013*. New York, New York, USA: ACM Press, 595–604. doi:10.1145/2506583.2506643.
- Huang, Shunping, James Holt, Chia-Yu Kao, Leonard McMillan, and Wei Wang. 2014. “A Novel Multi-Alignment Pipeline for High-Throughput Sequencing Data.” *Database* 2014. doi:10.1093/database/bau057.
- Ideraabdullah, Folami Y, Sebastien Vigneau, and Marisa S Bartolomei. 2008. “Genomic Imprinting Mechanisms in Mammals.” *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* 647 (1-2): 77–85. doi:10.1016/j.mrfmmm.2008.08.008.
- Ishikawa, Hitoshi, Aine Rattigan, Reinald H Fundele, and Paul S Burgoyne. 2003. “Effects of Sex Chromosome Dosage on Placental Size in Mice.” *Biology of Reproduction* 69 (2): 483–88. doi:10.1095/biolreprod.102.012641.
- Josefsson, Caroline, Brian Dilkes, and Luca Comai. 2006. “Parent-Dependent Loss of Gene Silencing During Interspecies Hybridization.” *Current Biology* 16 (13): 1322–28. doi:10.1016/j.cub.2006.05.045.
- Judson, H, B E Hayward, E Sheridan, and D T Bonthron. 2002. “A Global Disorder of Imprinting in the Human Female Germ Line.” *Nature* 416: 539–42.

- Jullien, Pauline E, and Frédéric Berger. 2010. "Parental Genome Dosage Imbalance Deregulates Imprinting in *Arabidopsis*." Edited by Joseph R Ecker. *PLoS Genetics* 6 (3): e1000885. doi:10.1371/journal.pgen.1000885.
- Kaneuchi, Masanori, Masahiro Sasaki, Yuichiro Tanaka, Hiroaki Shiina, Hideto Yamada, Ritsu Yamamoto, Noriaki Sakuragi, Hideki Enokida, Mukesh Verma, and Rajvir Dahiya. 2005. "*Wt1* And *Wt1-as* Genes Are Inactivated by Promoter Methylation in Ovarian Clear Cell Adenocarcinoma." *Cancer* 104 (9): 1924–30. doi:10.1002/cncr.21397.
- Kay, G F, G D Penny, D Patel, A Ashworth, and N Brockdorff. 1993. "Expression of Xist During Mouse Development Suggests a Role in the Initiation of X Chromosome Inactivation." *Cell* 72: 171–82.
- Kishino, T, M Lalande, and J Wagstaff. 1997. "*Ube3a*/E6-AP Mutations Cause Angelman Syndrome." *Nature Genetics* 15 (4): 70–73. doi:10.1038/ng0497-411d.
- Kropáčková, L, J Pialek, V Gergelits, J Forejt, and R Reifová. 2015. "Maternal-Foetal Genomic Conflict and Speciation: No Evidence for Hybrid Placental Dysplasia in Crosses Between Two House Mouse Subspecies." *Journal of Evolutionary Biology* 28 (3): 1–11. doi:10.1111/jeb.12602.
- Kurz, H, U Zechner, Annie Orth, and Reinald H Fundele. 1999. "Lack of Correlation Between Placenta and Offspring Size in Mouse Interspecific Crosses." *Anatomy and Embryology* 200 (3): 335–43. doi:10.1007/s004290050284.



- Latham, K E. 1996. "X Chromosome Imprinting and Inactivation in the Early Mammalian Embryo." *Trends in Genetics* 12 (4): 134–38.
- Liao, Yang, Gordon K Smyth, and Wei Shi. 2014. "featureCounts: an Efficient General Purpose Program for Assigning Sequence Reads to Genomic Features." *Bioinformatics* 30 (7): 923–30. doi:10.1093/bioinformatics/btt656.
- Lifschytz, E, and D L Lindsley. 1972. "The Role of X-Chromosome Inactivation During Spermatogenesis." *Proceedings of the National Academy of Sciences* 69 (1): 182–86.
- Lim, Derek Hock Kiat, and Eamonn Richard Maher. 2010. "Genomic Imprinting Syndromes and Cancer." *Advances in Genetics* 70: 145–75. doi:10.1016/B978-0-12-380866-0.60006-X.
- Loschiavo, Maria, Quang K Nguyen, Amanda R Duselis, and Paul B Vrana. 2007. "Mapping and Identification of Candidate Loci Responsible for *Peromyscus* Hybrid Overgrowth." *Mammalian Genome* 18 (1): 75–85. doi:10.1007/s00335-006-0083-x.
- Malik, K, A Salpekar, A Hancock, K Moorwood, S Jackson, A Charles, and K W Brown. 2000. "Identification of Differential Methylation of the *Wt1* Antisense Regulatory Region and Relaxation of Imprinting in Wilms' Tumor." *Cancer Research* 60 (9): 2356–60.
- Mallarino, R, O Campàs, J A Fritz, Kevin J Burns, Olivia G Weeks, Michael P Brenner, and Arhat Abzhanov. 2012. "Closely Related Bird Species Demonstrate Flexibility Between Beak Morphology and Underlying Developmental Programs." *Proceedings of the National Academy of Sciences* 109 (40): 16222–27.

- Mann, Jeffrey R, Pirooska E Szabo, Michael R Reed, and Judith Singer-Sam. 2000. "Methylated DNA Sequences in Genomic Imprinting." *Critical Reviews in Eukaryotic Gene Expression* 10 (3-4): 1–18.
- Martin, Marcel. 2011. "Cutadapt Removes Adapter Sequences From High-Throughput Sequencing Reads." *EMBnet.Journal* 17 (July): 1–3.
- McCarthy, D J, Y Chen, and G K Smyth. 2012. "Differential Expression Analysis of Multifactor RNA-Seq Experiments with Respect to Biological Variation." *Nucleic Acids Research* 40 (10): 4288–97. doi:10.1093/nar/gks042.
- McKenna, A, M Hanna, E Banks, A Sivachenko, K Cibulskis, A Kernytsky, K Garimella, et al. 2010. "The Genome Analysis Toolkit: a MapReduce Framework for Analyzing Next-Generation DNA Sequencing Data." *Genome Research* 20 (9): 1297–1303. doi:10.1101/gr.107524.110.
- Michalak, P. 2003. "Genome-Wide Patterns of Expression in Drosophila Pure Species and Hybrid Males." *Molecular Biology and Evolution* 20 (7): 1070–76. doi:10.1093/molbev/msg119.
- Monk, David, Alexandre Wagschal, Philippe Arnaud, Pari-Sima Müller, Layla Parker-Katiraei, Déborah Bourc'h, Stephen W Scherer, Robert Feil, Philip Stanier, and Gudrun E Moore. 2008. "Comparative Analysis of Human Chromosome 7q21 and Mouse Proximal Chromosome 6 Reveals a Placental-Specific Imprinted Gene, *Tfpi2/Tfpi2*, Which Requires EHMT2 and EED for Allelic-Silencing." *Genome Research* 18 (8): 1270–81. doi:10.1101/gr.077115.108.

- Moore, Tom, and D Haig. 1991. "Genomic Imprinting in Mammalian Development: a Parental Tug-of-War." *Trends in Genetics* 7 (2): 45–49. doi:10.1016/0168-9525(91)90230-N.
- Morison, Ian M, Joshua P Ramsay, and Hamish G Spencer. 2005. "A Census of Mammalian Imprinting." *Trends in Genetics* 21 (8): 457–65. doi:10.1016/j.tig.2005.06.008.
- Muller, H J. 1942. "Isolating Mechanisms, Evolution, and Temperature." *Biological Symposia* 6: 71–125.
- Nadaf, Al, Shafagh, Janine E Deakin, Clément Gilbert, Terence J Robinson, Jennifer A M Graves, and Paul D Waters. 2012. "A Cross-Species Comparison of Escape From X Inactivation in Eutheria: Implications for Evolution of X Chromosome Inactivation." *Chromosoma* 121 (1): 71–78. doi:10.1007/s00412-011-0343-8.
- Newell-Price, J, AJL Clark, and P King. 2000. "DNA Methylation and Silencing of Gene Expression." *Trends in Endocrinology & Metabolism* 11 (4): 142–47. doi:10.1016/s1043-2760(00)00248-4.
- Nicholls, R D, and J L Knepper. 2001. "Genome Organization, Function, and Imprinting in Prader-Willi and Angelman Syndromes." *Annual Review of Genomics and Human Genetics* 2 (1): 153–75. doi:10.1146/annurev.genom.2.1.153.
- Nishita, Y, I Yoshida, T Sado, and N Takagi. 1996. "Genomic Imprinting and Chromosomal Localization of the Human *Mest* Gene." *Genomics* 36 (3): 539–42. doi:10.1006/geno.1996.0502.

- Ortíz-Barrientos, Daniel, Brian A Counterman, and Mohamed A F Noor. 2006. "Gene Expression Divergence and the Origin of Hybrid Dysfunctions." *Genetica* 129 (1): 71–81. doi:10.1007/s10709-006-0034-1.
- Pollard, Katherine S, David Serre, Xu Wang, Heng Tao, Elin Grundberg, Thomas J Hudson, Andrew G Clark, and Kelly Frazer. 2007. "A Genome-Wide Approach to Identifying Novel-Imprinted Genes" 122 (6): 625–34. doi:10.1007/s00439-007-0440-1.
- Preibisch, S, S Saalfeld, and P Tomancak. 2009. "Globally Optimal Stitching of Tiled 3D Microscopic Image Acquisitions." *Bioinformatics* 25 (11): 1463–65. doi:10.1093/bioinformatics/btp184.
- Proudhon, Charlotte, and Déborah Bourc'h. 2010. "Identification and Resolution of Artifacts in the Interpretation of Imprinted Gene Expression." *Briefings in Functional Genomics* 9 (5-6): 374–84. doi:10.1093/bfpg/elq020.
- Prud'homme, Benjamin, Nicolas Gompel, and Sean B Carroll. 2007. "Emerging Principles of Regulatory Evolution." *Proceedings of the National Academy of Sciences* 104 (suppl. 1): 8605–12. doi:10.1073/pnas.0700488104.
- Reik, Wolf, and E R Maher. 1997. "Imprinting in Clusters: Lessons From Beckwith-Wiedemann Syndrome." *Trends in Genetics* 13 (8): 330–34.
- Reik, Wolf, Miguel Constancia, Abigail Fowden, Neil Anderson, Wendy Dean, Anne Ferguson-Smith, Benjamin Tycko, and Colin Sibley. 2003. "Regulation of Supply and Demand for Maternal Nutrients in Mammals by Imprinted Genes." *The Journal of*

- Physiology* 547 (Pt 1): 35–44. doi:10.1113/jphysiol.2002.033274.
- Renaut, S, and L Bernatchez. 2010. “Transcriptome-Wide Signature of Hybrid Breakdown Associated with Intrinsic Reproductive Isolation in Lake Whitefish Species Pairs (*Coregonus* Spp. Salmonidae).” *Heredity* 106 (6): 1003–11. doi:10.1038/hdy.2010.149.
- Robinson, M D, D J McCarthy, and G K Smyth. 2010. “edgeR: a Bioconductor Package for Differential Expression Analysis of Digital Gene Expression Data.” *Bioinformatics* 26 (1): 139–40. doi:10.1093/bioinformatics/btp616.
- Rodrigues, Jessica A, and Daniel Zilberman. 2015. “Evolution and Function of Genomic Imprinting in Plants.” *Genes & Development* 29 (24). Cold Spring Harbor Lab: 2517–31. doi:10.1101/gad.269902.115.
- Rollin, J, S Iochmann, C Bléchet, F Hubé, S Régina, S Guyétant, E Lemarié, P Reverdiau, and Y Gruel. 2005. “Expression and Methylation Status of Tissue Factor Pathway Inhibitor-2 Gene in Non-Small-Cell Lung Cancer.” *British Journal of Cancer* 92 (4): 775–83. doi:10.1038/sj.bjc.6602298.
- Safronova, L D, and N Y Vasil'eva. 1996. “Meiotic Abnormalities in Interspecific Hybrids Between *Phodopus Sungorus* (Pallas, 1773) and *Ph. Campbelli* (Thomas, 1905).” *Russian Journal of Genetics* 32 (4). Moscow, Russia: MAIK Nauka/Interperiodica Pub.; New York: Distributed by Plenum/Consultants Bureau, c1994-: 486–94.
- Safronova, L D, E V Cherepanova, and N Y Vasil'eva. 1999. “Specific Features of the First

- Meiotic Division in Hamster Hybrids Obtained by Backcrossing *Phodopus Sungorus* And *Phodopus Campbelli*." *Russian Journal of Genetics* 35 (2): 184–88.
- Sanchez-Delgado, Marta, Alejandro Martin-Trujillo, Chiharu Tayama, Enrique Vidal, Manel Esteller, Isabel Iglesias-Platas, Nandita Deo, et al. 2015. "Absence of Maternal Methylation in Biparental Hydatidiform Moles From Women with NLRP7 Maternal-Effect Mutations Reveals Widespread Placenta-Specific Imprinting." Edited by Thomas Eggermann. *PLoS Genetics* 11 (11): e1005644–17.  
doi:10.1371/journal.pgen.1005644.
- Sapienza, Carmen. 1989. "Genome Imprinting and Dominance Modification." *Annals of the New York Academy of Sciences* 564 (July): 24–38. doi:10.1111/j.1749-6632.1989.tb25885.x.
- Saukkonen, T. 2004. "Dose-Dependent Effects of Recombinant Human Insulin-Like Growth Factor (IGF)-I/IGF Binding Protein-3 Complex on Overnight Growth Hormone Secretion and Insulin Sensitivity in Type 1 Diabetes." *Journal of Clinical Endocrinology & Metabolism* 89 (9): 4634–41. doi:10.1210/jc.2004-0243.
- Scarpino, S V, P J Hunt, F J Garcia-De-Leon, T E Juenger, M Scharl, and M Kirkpatrick. 2013. "Evolution of a Genetic Incompatibility in the Genus *Xiphophorus*." *Molecular Biology and Evolution* 30 (10): 2302–10. doi:10.1093/molbev/mst127.
- Schneider, Caroline A, Wayne S Rasband, and Kevin W Eliceiri. 2012. "NIH Image to ImageJ: 25 Years of Image Analysis." *Nature Methods* 9 (7): 671–75.  
doi:10.1038/nmeth.2089.

- Schütt, S, A R Florl, W Shi, M C Hemberger, and Annie Orth. 2003. "DNA Methylation in Placentas of Interspecies Mouse Hybrids." *Genetics* 165: 223–28.
- Scribner, S J, and K E Wynne-Edwards. 1994. "Disruption of Body Temperature and Behavior Rhythms During Reproduction in Dwarf Hamsters (*Phodopus*)." *Physiology & Behavior* 55 (2): 361–69.
- Shapiro, M D, M E Marks, C L Peichel, B K Blackman, K S Nereng, B Jónsson, D Schluter, and D M Kingsley. 2004. "Genetic and Developmental Basis of Evolutionary Pelvic Reduction in Threespine Sticklebacks." *Nature* 428 (6984): 717–23.
- Shi, Wei, Antje Krella, Annie Orth, Yang Yu, and Reinald H Fundele. 2005. "Widespread Disruption of Genomic Imprinting in Adult Interspecies Mouse (*Mus*) Hybrids" 43 (3): 100–108. doi:10.1002/gene.20161.
- Shi, Wei, Louis Lefebvre, Yang Yu, Sabine Otto, Antje Krella, Annie Orth, and Reinald H Fundele. 2004. "Loss-of-Imprinting of *Peg1* In Mouse Interspecies Hybrids Is Correlated with Altered Growth" 39 (1): 65–72. doi:10.1002/gene.20027.
- Sokolov, V E, and N I Vasil'eva. 1993. "Hybridologic Analysis Confirms the Species Specificity of *Phodopus Sungorus* (Pallus, 1773) and *Phodopus Campbelli* (Thomas, 1905)." *Doklady Akademii Nauk* 332 (1): 120–23.
- Solter, D. 1988. "Differential Imprinting and Expression of Maternal and Paternal Genomes." *Annual Review of Genetics* 22 (1): 127–46.  
doi:10.1146/annurev.ge.22.120188.001015.

- Spencer, Hamish G, and Andy G Clark. 2014. "Non-Conflict Theories for the Evolution of Genomic Imprinting." *Heredity* 113 (2). Nature Publishing Group: 112–18.  
doi:10.1038/hdy.2013.129.
- Sultan, Marc, Simon Dökel, Vyacheslav Amstislavskiy, Daniela Wuttig, Holger Sülthmann, Hans Lehrach, and Marie-Laure Yaspo. 2012. "A Simple Strand-Specific RNA-Seq Library Preparation Protocol Combining the Illumina TruSeq RNA and the dUTP Methods." *Biochemical and Biophysical Research Communications* 422 (4): 643–46.  
doi:10.1016/j.bbrc.2012.05.043.
- Takada, Hisashi, Naoki Wakabayashi, Osamu Dohi, Kohichiroh Yasui, Chouhei Sakakura, Shoji Mitsufuji, Masafumi Taniwaki, and Toshikazu Yoshikawa. 2010. "Tissue Factor Pathway Inhibitor 2 (*Tfpi2*) Is Frequently Silenced by Aberrant Promoter Hypermethylation in Gastric Cancer." *Cancer Genetics and Cytogenetics* 197 (1): 16–24. doi:10.1016/j.cancergencyto.2009.11.004.
- Theiler, K. 1972. *The House Mouse. Development and Normal Stages From Fertilization to 4 Weeks of Age*. New York, NY: Springer-Verlag.
- Todd, Erica V, Michael A Black, and Neil J Gemmell. 2016. "The Power and Promise of RNA-Seq in Ecology and Evolution." *Molecular Ecology* 25 (6): 1224–41.  
doi:10.1111/mec.13526.
- Trivers, R L. 1974. "Parent-Offspring Conflict." *Integrative and Comparative Biology* 14 (1):249–64. doi:10.1093/icb/14.1.249.



- Turan, Nahid, Sunita Katari, Leigh F Gerson, Raffi Chalian, Michael W Foster, John P Gaughan, Christos Coutifaris, and Carmen Sapienza. 2010. "Inter- and Intra-Individual Variation in Allele-Specific Dna Methylation and Gene Expression in Children Conceived Using Assisted Reproductive Technology." Edited by Jeannie T Lee. *PLoS Genetics* 6 (7): e1001033–14. doi:10.1371/journal.pgen.1001033.
- Turner, Leslie M, Michael A White, Diethard Tautz, and Bret A Payseur. 2014. "Genomic Networks of Hybrid Sterility." Edited by John H Willis. *PLoS Genetics* 10 (2): e1004162. doi:10.1371/journal.pgen.1004162.s008.
- Van der Auwera, Geraldine A, Mauricio O Carneiro, Chris Hartl, Ryan Poplin, Guillermo del Angel, Ami Levy-Moonshine, Tadeusz Jordan, et al. 2013. "From FastQ Data to High Confidence Variant Calls: the Genome Analysis Toolkit Best Practices Pipeline." *Current Protocols in Bioinformatics / Editorial Board, Andreas D. Baxeavanis, Et Al.* 43: 11–33. doi:10.1002/0471250953.bi1110s43.
- Varmuza, S. 1993. "Gametic Imprinting as a Speciation Mechanism in Mammals." *Journal of Theoretical Biology* 164 (1): 1–13. doi:10.1006/jtbi.1993.1137.
- Varmuza, S, and M Mann. 1994. "Genomic Imprinting-Defusing the Ovarian Time Bomb." *Trends in Genetics* 10 (4): 118–23.
- Vrana, P B. 2007. "Genomic Imprinting as a Mechanism of Reproductive Isolation in Mammals." *Journal of Mammalogy* 88 (1): 5–23.
- Vrana, P B, J A Fossella, P Matteson, T del Rio, M J O'Neill, and S M Tilghman. 2000. "Genetic

- and Epigenetic Incompatibilities Underlie Hybrid Dysgenesis in *Peromyscus*." *Nature Genetics* 25 (1): 120–24. doi:10.1038/75518.
- Vrana, P B, X J Guan, R S Ingram, and S M Tilghman. 1998. "Genomic Imprinting Is Disrupted in Interspecific *Peromyscus* Hybrids." *Nature Genetics* 20 (4): 362–65. doi:10.1038/3833.
- Vrana, Paul, Paul Matteson, Jennifer Schmidt, Robert Ingram, Andrew Joyce, Kelly Prince, Michael Dewey, and Shirley Tilghman. 2001. "Genomic Imprinting of a Placental Lactogen Gene in *Peromyscus*." *Development Genes and Evolution* 211 (11): 523–32. doi:10.1007/s00427-001-0188-x.
- Wake, N, N Takagi, and M Sasaki. 1976. "Non-Random Inactivation of X Chromosome in the Rat Yolk Sac." *Nature* 262: 580–81.
- Wang, Xu, and Andy G Clark. 2014. "Using Next-Generation RNA Sequencing to Identify Imprinted Genes." *Heredity* 113 (2): 156–66. doi:10.1038/hdy.2014.18.
- Wang, Xu, Donald C Miller, Rebecca Harman, Douglas F Antczak, and Andrew G Clark. 2013. "Paternal Expressed Genes Predominate in the Placenta." *Proceedings of the National Academy of Sciences of the United States of America* 110 (26): 10705–10. doi:10.1073/pnas.1308998110.
- Wang, Xu, Paul D Soloway, and Andrew G Clark. 2011. "A Survey for Novel Imprinted Genes in the Mouse Placenta by mRNA-Seq." *Genetics* 189 (1): 109–22. doi:10.1534/genetics.111.130088.

- Wang, Xu, Qi Sun, Sean D McGrath, Elaine R Mardis, Paul D Soloway, and Andrew G Clark. 2008. "Transcriptome-Wide Identification of Novel Imprinted Genes in Neonatal Mouse Brain." *PLoS ONE* 3 (12): e3839–39. doi:10.1371/journal.pone.0003839.
- Wei, Xiaoxing, and Xiangfeng Wang. 2013. "A Computational Workflow to Identify Allele-Specific Expression and Epigenetic Modification in Maize." *Genomics, Proteomics & Bioinformatics* 11 (4). Beijing Institute of Genomics, Chinese Academy of Sciences and Genetics Society of China: 247–52. doi:10.1016/j.gpb.2013.05.006.
- Weksberg, Rosanna, Cheryl Shuman, and J Bruce Beckwith. 2010. "Beckwith-Wiedemann Syndrome." *European Journal of Human Genetics* 18 (1): 8–14. doi:10.1038/ejhg.2009.106.
- Wiley, C D, H H Matundan, Amanda R Duselis, and A T Isaacs. 2008. "Patterns of Hybrid Loss of Imprinting Reveal Tissue-and Cluster-Specific Regulation." *PLoS ONE* 3 (10): 1–10. doi:10.1371/journal.pone.0003572.t001.
- Wolf, Jason B, and R Hager. 2006. "A Maternal–Offspring Coadaptation Theory for the Evolution of Genomic Imprinting." *PLoS Biology* 4 (12): 2238–43. doi:10.1371/journal.pbio.0040380.st001.
- Wolf, Jason B, J Lindell, and N Backstrom. 2010. "Speciation Genetics: Current Status and Evolving Approaches" 365 (1547): 1717–33. doi:10.1098/rstb.2010.0023.
- Wolff, P, H Jiang, G Wang, J Santos-González, and C Köhler. 2015. "Paternaly Expressed Imprinted Genes Establish Postzygotic Hybridization Barriers in *Arabidopsis*

*Thaliana*" 4: e1000605. doi:10.7554/elife.10074.

- Wutz, Anton. 2011. "Gene Silencing in X-Chromosome Inactivation: Advances in Understanding Facultative Heterochromatin Formation." *Nature Reviews Genetics* 12 (8): 542–53. doi:10.1038/nrg3035.
- Yang, Fan, Tomas Babak, Jay Shendure, and Christine M Disteche. 2010. "Global Survey of Escape From X Inactivation by RNA-Sequencing in Mouse." *Genome Research* 20 (5): 614–22. doi:10.1101/gr.103200.109.
- Zechner, U, M C Hemberger, Miguel Constanica, Annie Orth, Ioannis Dragatsis, Angela Lüttges, Horst Hameister, and Reinald H Fundele. 2002. "Proliferation and Growth Factor Expression in Abnormally Enlarged Placentas of Mouse Interspecific Hybrids." *Developmental Dynamics* 224 (2): 125–34. doi:10.1002/dvdy.10094.
- Zechner, U, M Reulez, Annie Orth, and F Bonhomme. 1996. "An X-Chromosome Linked Locus Contributes to Abnormal Placental Development in Mouse Interspecific Hybrids." *Nature Genetics* 12 (4): 398–403. doi:10.1038/ng0496-398.
- Zechner, U, Matthais Reule, P S Burgoyne, A Schubert, Annie Orth, H Hameister, and Reinald H Fundele. 1997. "Paternal Transmission of X-Linked Placental Dysplasia in Mouse Interspecific Hybrids." *Genetics* 146 (4). Genetics Soc America: 1399–1405.
- Zechner, U, W Shi, M C Hemberger, H Himmelbauer, Sabine Otto, Annie Orth, V Kalscheuer, et al. 2004. "Divergent Genetic and Epigenetic Post-Zygotic Isolation Mechanisms in *Mus* And *Peromyscus*." *Journal of Evolutionary Biology* 17 (2): 453–60.

doi:10.1046/j.1420-9101.2003.00656.x.

Figure 1

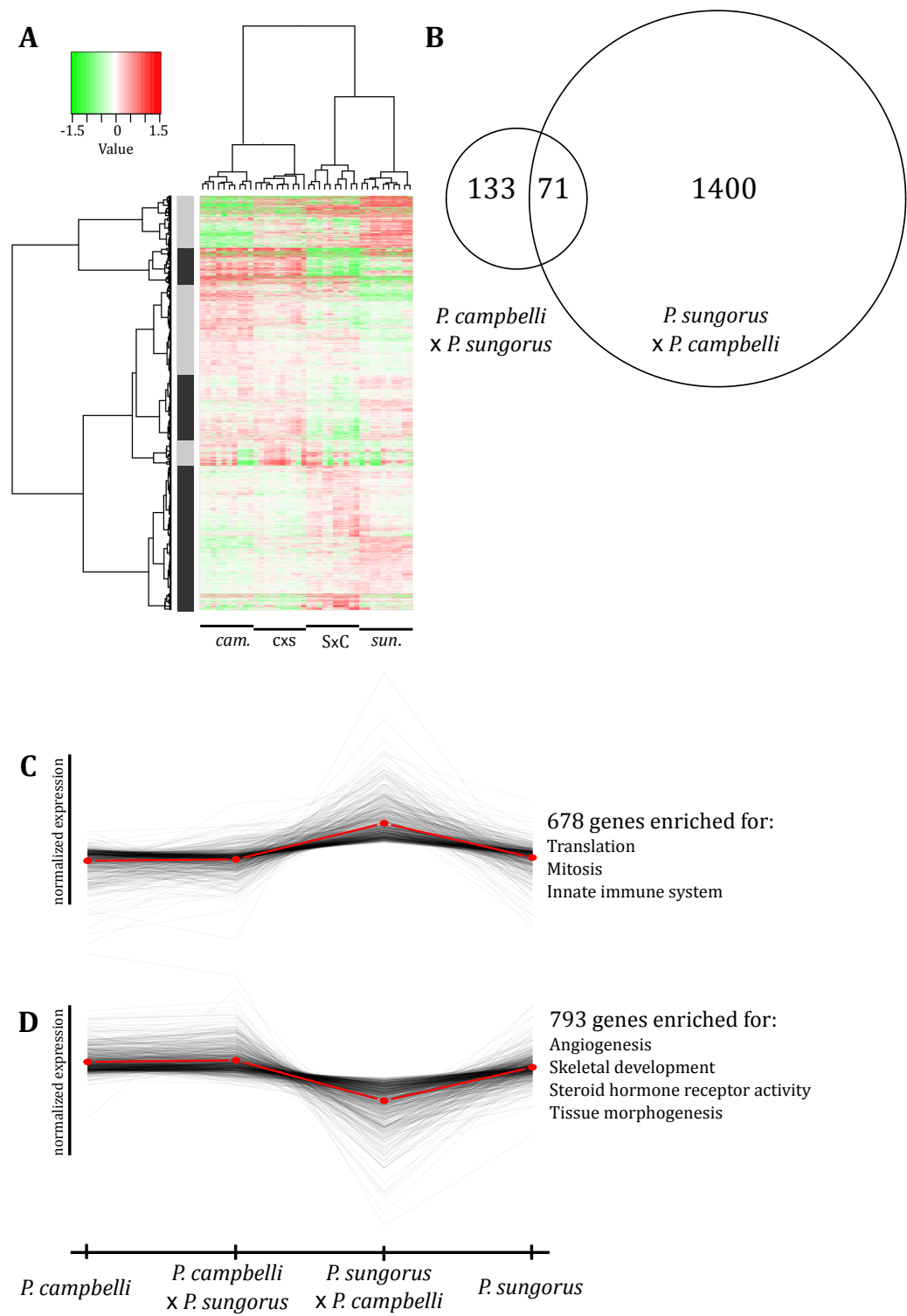


Figure 2

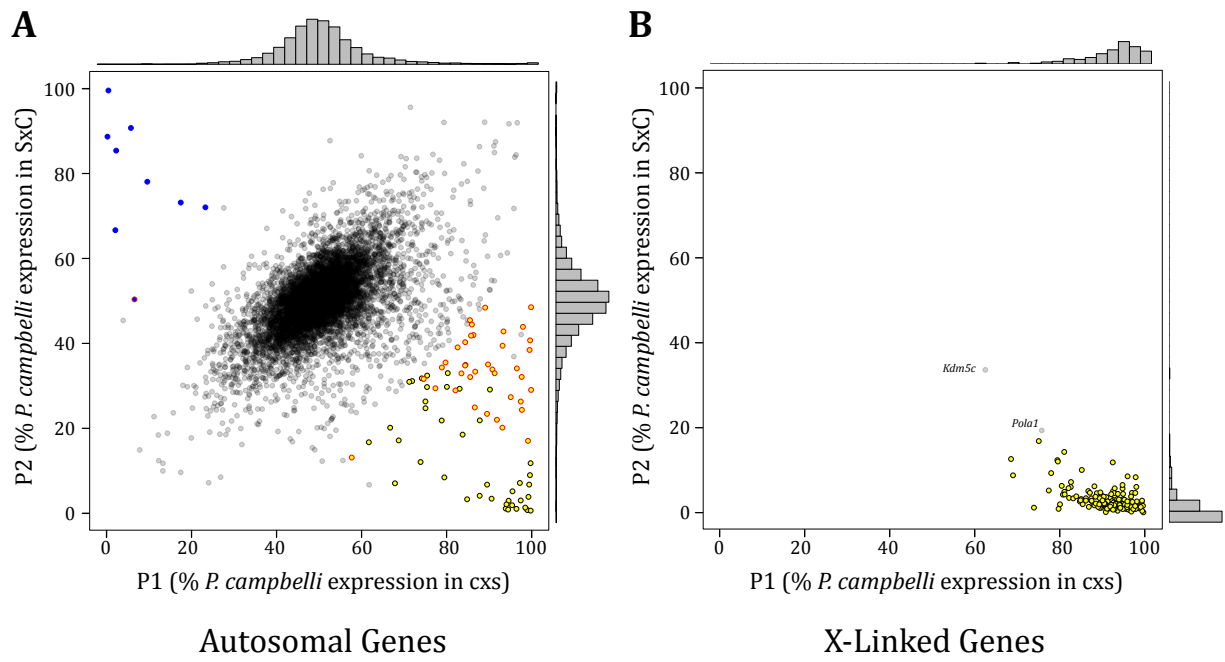


Figure 3

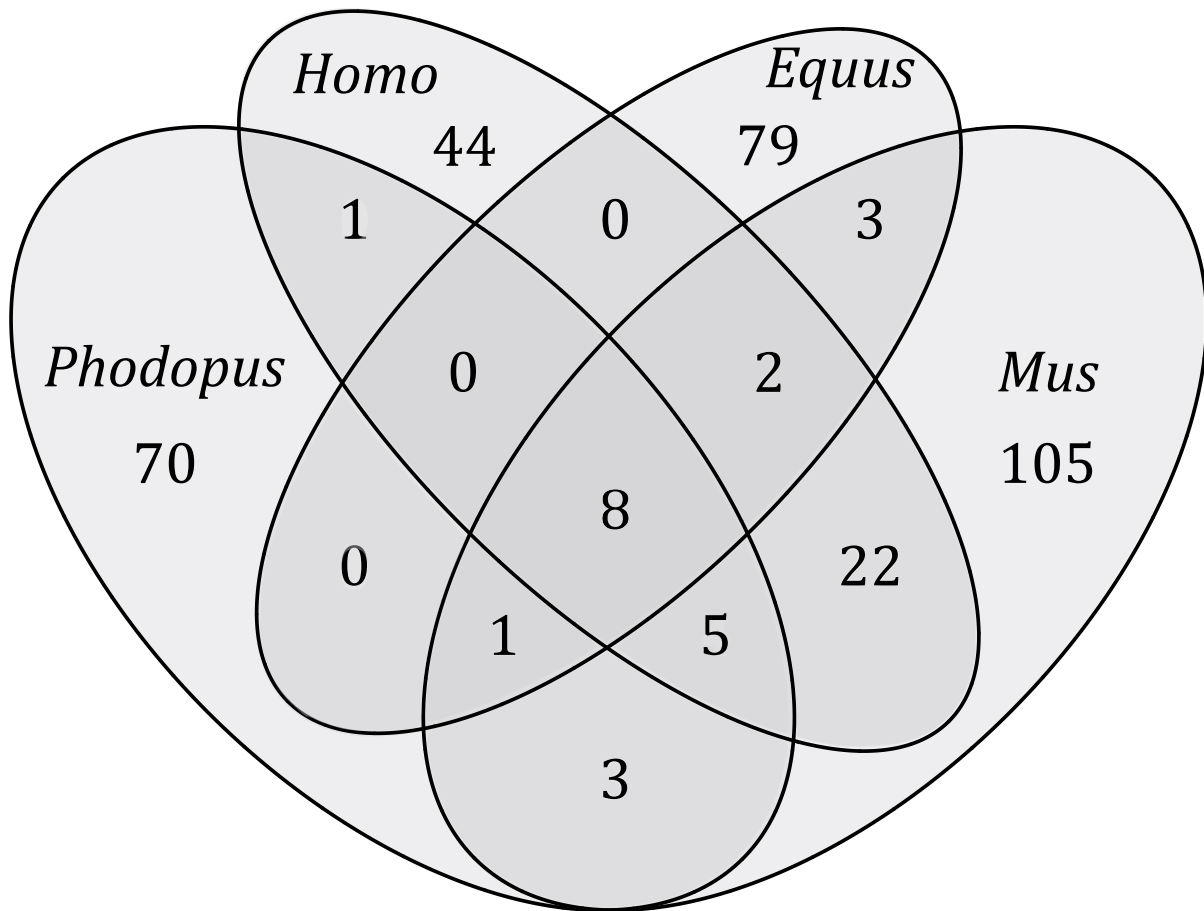




Figure 4

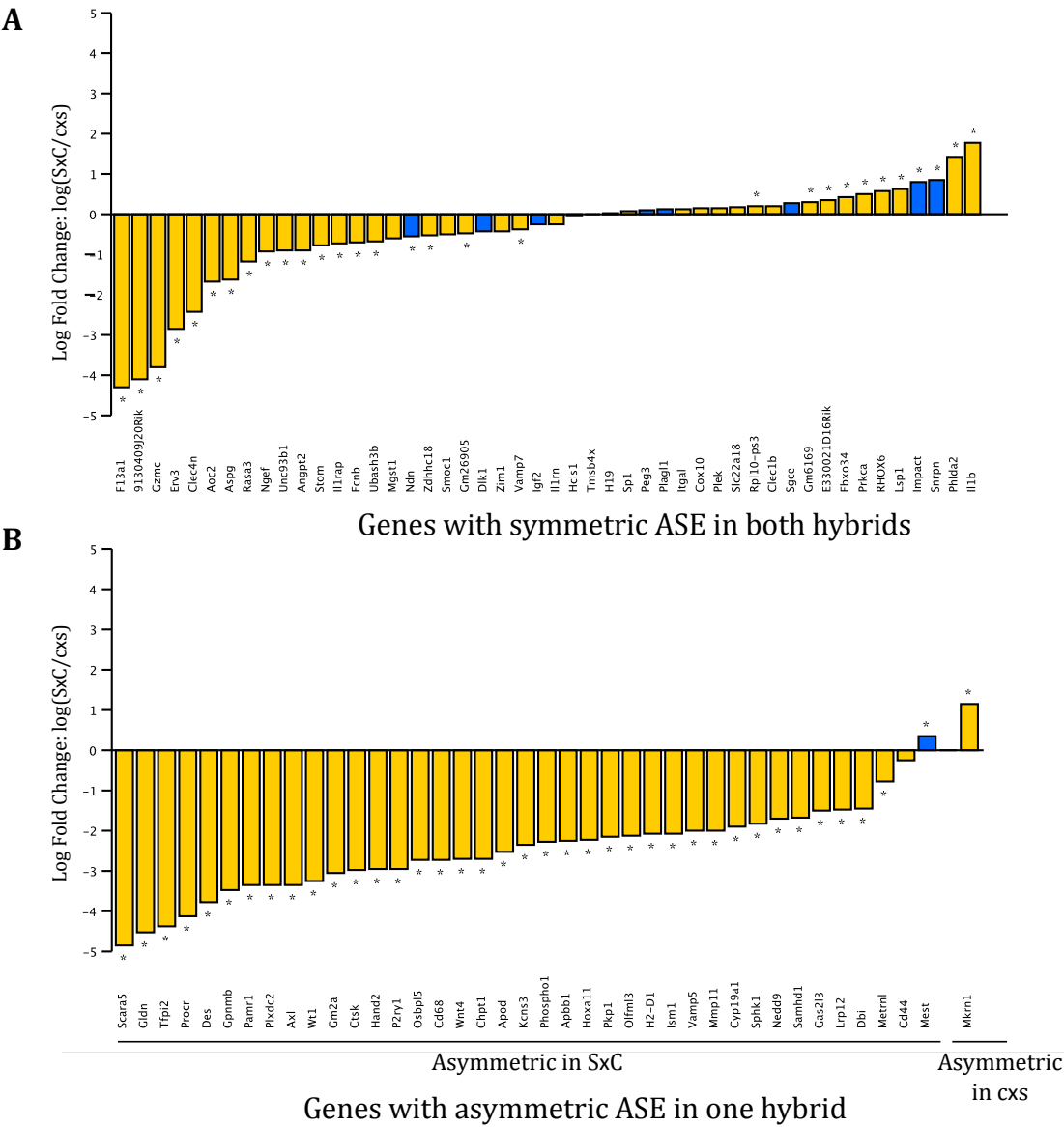
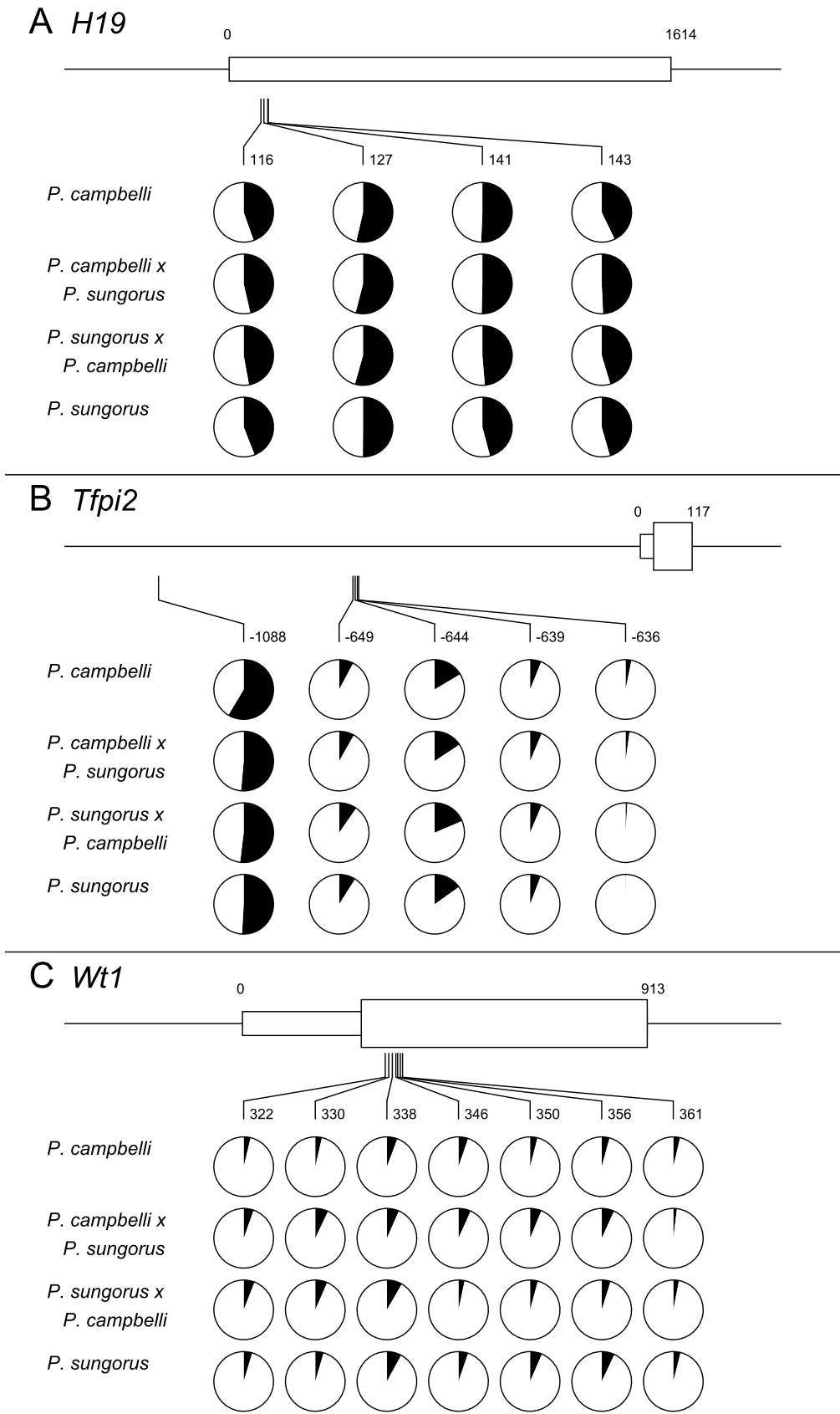
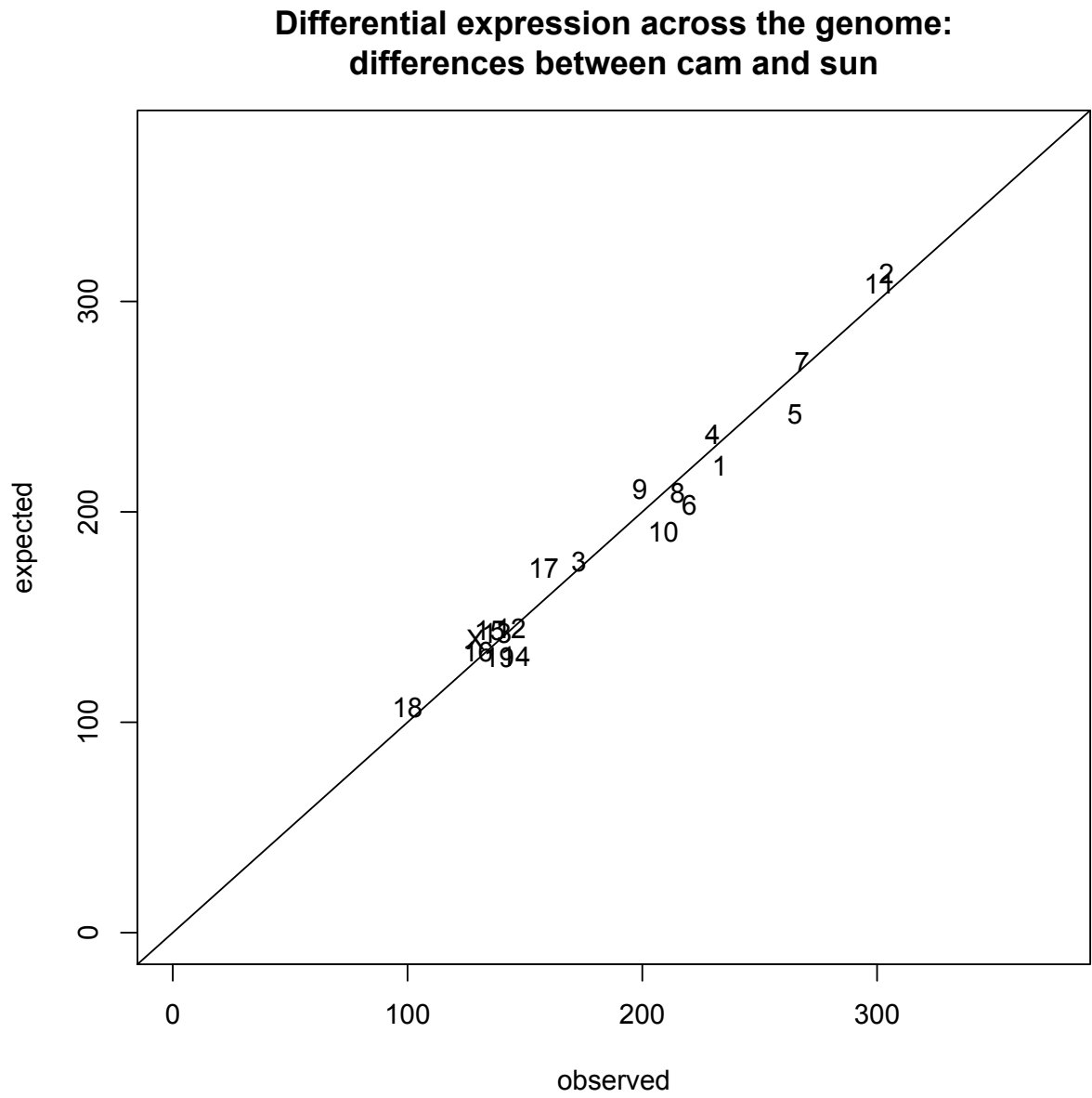


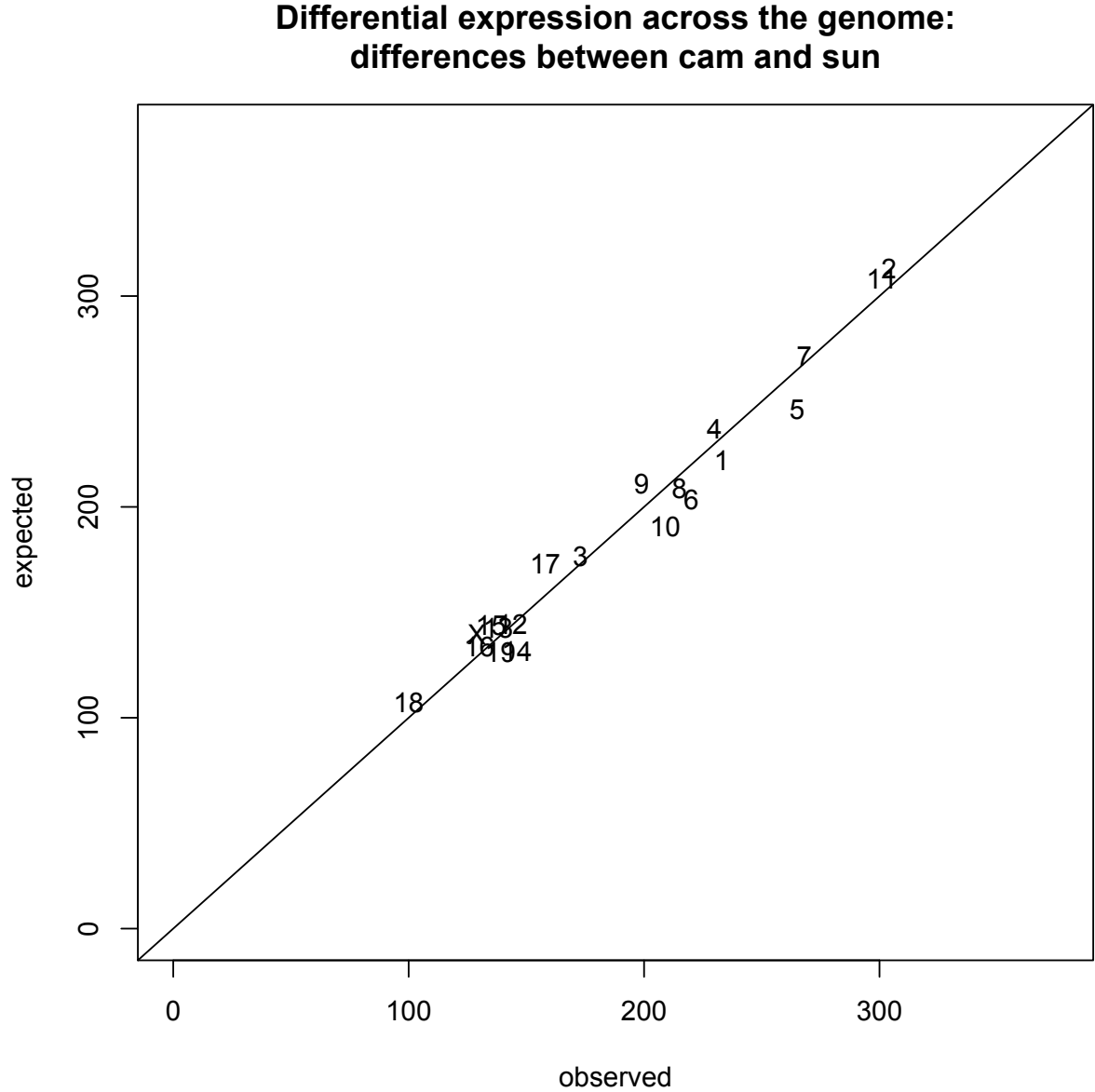
Figure 5



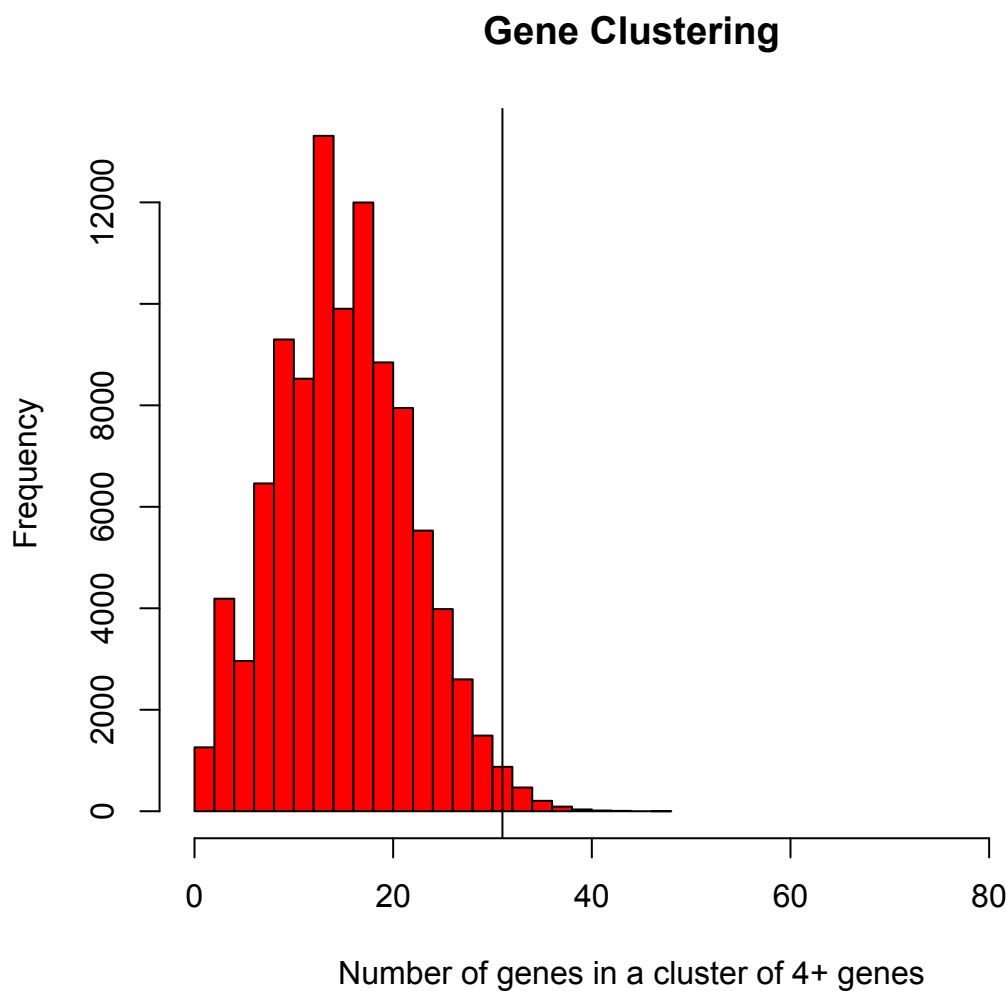
Supplemental Figure 1A



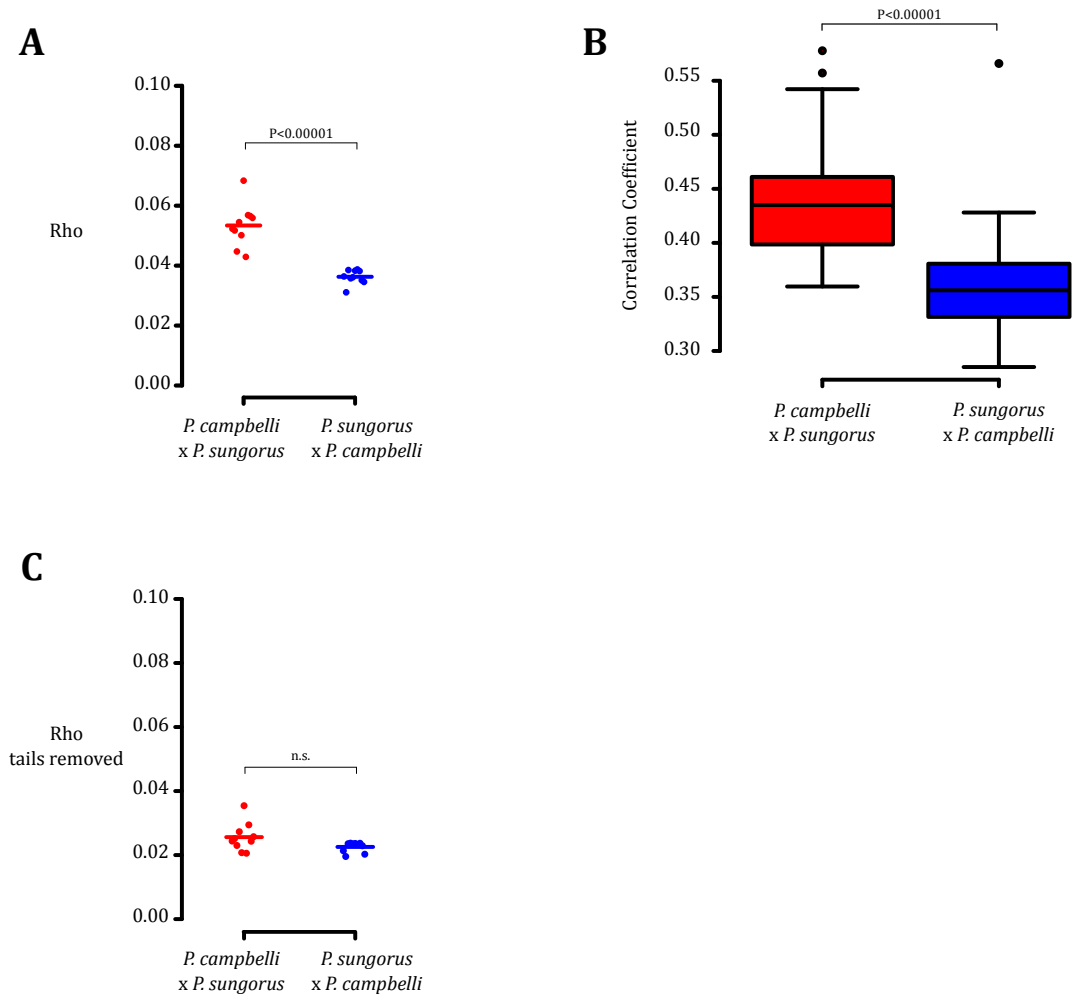
Supplemental Figure 1B



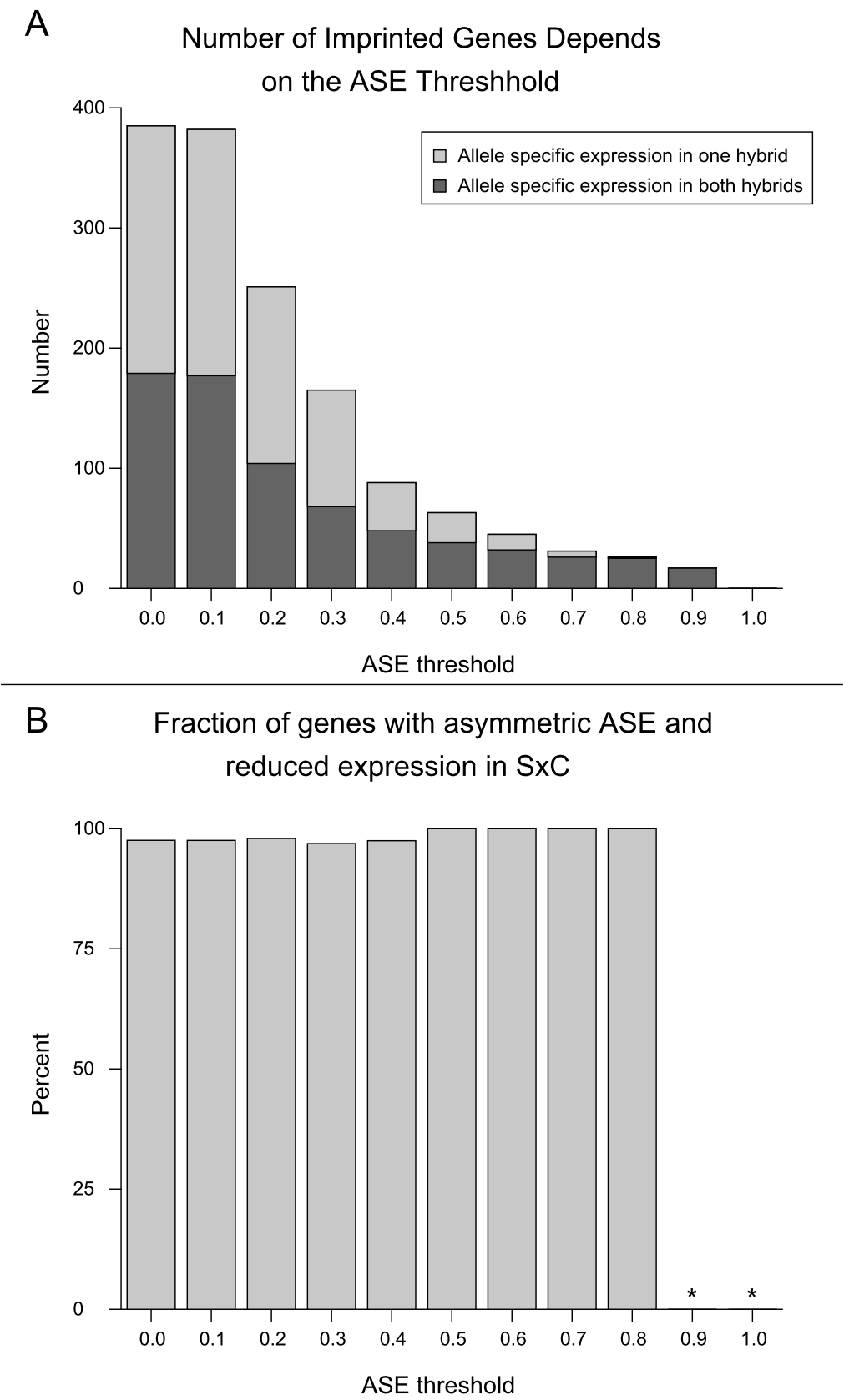
Supplemental Figure 2



Supplemental Figure 3



Supplemental Figure 4



Supplemental Table 1A: DAVID enrichment for DE genes between *P. campbelli* and *P. sungrou*s

Enrichment Group	Enrichment Score	Summary Term	Enrichment Terms
1	5.14	Innate Immunity	acute inflammatory response response to wounding defense response inflammatory response innate immune response
2	3.39	Carbohydrate binding	carbohydrate binding polysaccharide binding pattern binding glycosaminoglycan binding heparin binding
3	2.56	Cognition	neurological system process cognition sensory perception
4	2.47	Cell signalling	cell-cell signalling synaptic transmission transmission of nerve impulse
5	2.25	Enzyme inhibitor activity	serine-type endopeptidase inhibitor activity endopeptidase inhibitor activity peptidase inhibitor activity enzyme inhibitor activity



Supplemental Table 1B: DAVID enrichment for DE genes between reciprocal hybrids

Enrichment Group	Enrichment Score	Summary Term	Enrichment Terms
1	5.31	Hormone regulation	regulation of hormone levels hormone metabolic process cellular hormone metabolic process
2	4.83	Mitosis	cell cycle process M phase cell cycle phase M phase of mitotic cell cycle mitosis nuclear division organelle fission mitotic cell cycle cell division cell cycle
3	3.41	Innate immune system	defense response response to wounding inflammatory response
4	3.19	Innate immune system	behavior locomotory behavior taxis chemotaxis cell chemotaxis leukocyte chemotaxis leukocyte migration neutrophil chemotaxis
5	2.96	Peptidase activity	endopeptidase activity peptidase activity peptidase activity, acting on L-amino acid peptides metallopeptidase activity proteolysis
6	2.71	Channel Activity	channel activity passive transmembrane transporter activity substrate specific channel activity ion channel activity cation channel activity metal ion transmembrane transporter activity ion transport gated channel activity metal ion transport cation transport potassium channel activity monovalent inorganic cation transport voltage-gated cation channel activity alkali metal ion binding voltage-gated potassium channel activity voltage-gated ion channel activity voltage-gated channel activity potassium ion transport potassium ion binding
7	2.7	Ion channel activity	ion channel activity cation channel activity gated channel activity ligand-gated ion channel activity ligand-gated channel activity extracellular ligand-gated ion channel activity neurotransmitter binding neurotransmitter receptor activity regulation of membrane potential
8	2.45	Peptidase activity	endopeptidase activity serine-type peptidase activity serine-type endopeptidase activity serine hydrolase activity
9	2.39	Carbohydrate binding	carbohydrate binding polysaccharide binding pattern binding glycosaminoglycan binding heparin binding
10	2.36	Nucleotide receptor activity	purinergic nucleotide receptor activity, G-protein coupled nucleotide receptor activity, G-protein coupled nucleotide receptor activity purinergic nucleotide receptor activity regulation of ion transport
11	2.35	Metabolic process	collagen catabolic process multicellular organismal catabolic process metalloendopeptidase activity collagen metabolic process multicellular organismal macromolecule metabolic process multicellular organismal metabolic process
12	2.28	Hormone binding	cellular hormone metabolic process steroid binding glucocorticoid metabolic process steroid metabolic process

Supplemental Table 1C: DAVID enrichment for DE genes between reciprocal hybrids that show high expression in SxC

Enrichment Group	Enrichment Score	Summary Term	Enrichment Terms
1	3.85	Innate immune system	immune response
			defense response
			innate immune response
2	3.13	Hormone Regulation	regulation of hormone levels
			hormone metabolic process
3	2.99	Channel activity	cellular hormone metabolic process
			channel activity
			passive transmembrane transporter activity
			gated channel activity
			substrate specific channel activity
			ion channel activity
			ion transport
			cation channel activity
			metal ion transmembrane transporter activity
			ligand-gated ion channel activity
			ligand-gated channel activity
			cation transport
			metal ion transport
			potassium channel activity
			voltage-gated cation channel activity
			monovalent inorganic cation transport
			voltage-gated ion channel activity
			voltage-gated channel activity
			voltage-gated potassium channel activity
			potassium ion transport
			alkali metal ion binding
			potassium ion binding
4	2.96	Nucleotide receptor activity	nucleotide receptor activity, G-protein coupled
			purinergic nucleotide receptor activity, G-protein coupled
			purinergic nucleotide receptor activity
5	2.73	Peptidase activity	nucleotide receptor activity
			metallopeptidase activity
			peptidase activity
			peptidase activity, acting on L-amino acid peptides
			endopeptidase activity
6	2.33	Ligand-gated ion channel activity	proteolysis
			ligand-gated ion channel activity
			ligand-gated channel activity
			extracellular ligand-gated ion channel activity
			neurotransmitter receptor activity
			neurotransmitter binding
6	2.33		regulation of membrane potential

Supplemental Table 1D: DAVID enrichment for DE genes between reciprocal hybrids that show low expression in SxC

Enrichment Group	Enrichment Score	Summary Term	Enrichment Terms
1	3.85	Innate immune system	immune response defense response innate immune response
2	3.13	Hormone regulation	regulation of hormone levels hormone metabolic process cellular hormone metabolic process
3	2.99	Channel Activity	channel activity passive transmembrane transporter activity gated channel activity substrate specific channel activity ion channel activity ion transport cation channel activity metal ion transmembrane transporter activity ligand-gated ion channel activity ligand-gated channel activity cation transport metal ion transport potassium channel activity voltage-gated cation channel activity monovalent inorganic cation transport voltage-gated ion channel activity voltage-gated channel activity voltage-gated potassium channel activity potassium ion transport alkali metal ion binding potassium ion binding
4	2.96	Nucleotide receptor activity	nucleotide receptor activity, G-protein coupled purinergic nucleotide receptor activity, G-protein coupled purinergic nucleotide receptor activity nucleotide receptor activity
5	2.73	Peptidase activity	metallopeptidase activity peptidase activity peptidase activity, acting on L-amino acid peptides endopeptidase activity proteolysis
6	2.33	Ion channel	ligand-gated ion channel activity ligand-gated channel activity extracellular ligand-gated ion channel activity neurotransmitter receptor activity

Supplemental Table 1E: DAVID enrichment for DE genes that show increased transgressive expression in SxC

Enrichment Group	Enrichment Score	Summary Term	Enrichment Terms
1	23.25	Translation	translation structural constituent of ribosome structural molecule activity
2	5.29	Mitosis	mitosis nuclear division M phase of mitotic cell cycle cell division organelle fission M phase mitotic cell cycle cell cycle phase cell cycle process cell cycle
3	4.97	Translation	translation factor activity, nucleic acid binding translational initiation translation initiation factor activity
4	2.26	Innate Immune system	cell chemotaxis leukocyte chemotaxis leukocyte migration neutrophil chemotaxis chemotaxis taxis locomotory behavior cell migration cell motility localization of cell cell motion behavior
5	2.24	Innate Immune system	defense response to bacterium defense response response to bacterium defense response to Gram-positive bacterium

Supplemental Table 1F: DAVID enrichment for DE genes that show reduced transgressive expression in SxC

Enrichment Group	Enrichment Score	Summary Term	Enrichment Terms
1	2.86	Angiogenesis	blood vessel development angiogenesis blood vessel morphogenesis vasculature development
2	2.59	Bone Development	skeletal system development bone development ossification
3	2.4	Hormone receptor activity	steroid hormone receptor activity ligand-dependent nuclear receptor activity steroid binding
4	2.38	Tissue morphogenesis	tube development tube morphogenesis gland morphogenesis morphogenesis of a branching structure epithelium development morphogenesis of an epithelium branching morphogenesis of a tube epithelial tube morphogenesis tissue morphogenesis

Supplemental Table 1G: DAVID enrichment for DE genes that show reduced transgressive expression in SxC

Enrichment Group	Enrichment Score	Summary Term	Enrichment Terms
1	2.27	Innate immunity	immune response defense response innate immune response immune effector process

### *Supplemental Text 1: Complexity in RNAseq libraries*

As Illumina libraries are prepared, many steps have the potential to bottleneck the nucleotide fragments and cause allelic dropout. This bottlenecking can drastically reduce the complexity of the sequencing library which can lead to artifacts and spurious ASE and will result in erroneously overcalling imprinted genes (DeVeale, van der Kooy, & Babak, 2012; Proudhon & Bourc'h, 2010; Wang & Clark, 2014). In order to test the complexity of our libraries, and following from the suggestions of Wang and Clark (2014), we define the variable P1 as the proportion of *P. campbelli* expression in c×s hybrids and P2 as the proportion of *P. campbelli* expression in S×C hybrids (Figure 2). Thus, evaluating library complexity becomes a matter modeling the frequency of allelic expression (P1 and P2) for all autosomal genes as a betabinomial distribution using R (v3.0.2, R Core Team, 2008). A betabinomial distribution is defined by two variables, mu (the average) and rho (the dispersion). Wang and Clark (2014) proposed that library complexity sufficient to estimate imprinting is achieved when rho is less than 0.050 and in examples of poorly prepared libraries, rho is near or above 0.2. We modeled rho for each library incorporating all genes with at least one diagnostic SNV which is approximately 70% (9,043 genes) of the 12,843 genes expressed in hamster placenta. Rho for our S×C libraries averages 0.036 with a range of 0.031-0.039. For c×s libraries, rho averages 0.053 with a range from 0.043-0.068 (Supplemental Figure 2a). Therefore, while our libraries are sufficiently complex enough to properly evaluate allele-specific expression, there is a treatment effect on library complexity (Student's T-test,  $P < 0.00001$ ).

The difference between the complexity of these libraries is likely due to true

biological differences rather than library preparation issues for four reasons. First, libraries were prepared in five groups of eight, with one replicate of each treatment prepared in tandem in order to avoid batch effects. Thus differences in rho can not be the result of a batch effect during library preparation.

Second, if low library complexity (high rho) is the result of poor library preparation of c×s libraries, then we would expect the correlation between maternal and paternal alleles to be higher in S×C libraries. This is because random allelic dropout due to a bottleneck will lower the correlation of ASE across c×s libraries as some lose the maternal allele, while others lose the paternal allele. Thus if bottlenecking strongly affects libraries, we expect to see high rho AND low correlation. Instead, we found that the opposite is actually true; c×s libraries have a higher inter-library correlation than do S×C libraries (Student's T-test,  $P < 0.00001$ , Supplemental Figure 2b), indicating that the differences in rho are not driven by bottlenecks, and are likely due to actual biology.

Third, a high rho value could be driven by the presence of imprinted genes. The betabinomial distribution assumes that 50% of alleles are maternally derived. As imprinted genes do not fit this assumption, their presence could be driving the increase in rho. There are over 40 imprinted genes which show significant ASE in c×s hybrids but not in S×C hybrids. These genes will act to increase the value of rho in c×s libraries but should actually keep rho low in S×C libraries. In order to test whether these genes artificially elevate rho in c×s libraries, we removed them and re-modeled rho. Specifically we removed all gene in the tails of the distribution of all libraries. Once the gene in the tails are removed, there is no longer a significant difference between rho for c×s libraries and S×C libraries (Students' T-test,  $P > 0.05$ , Supplemental Figure 2c). This demonstrates that the genes which show



asymmetric ASE do drive the treatment effects in rho and suggests that disrupted imprinting in S×C hybrids is an important and biologically real phenomenon.

Finally, as low library complexity is a function of the random bottlenecking of RNAs through the library preparation, we expect that an approximately equal number of genes should lose maternal alleles as lose {paternal alleles. We identified 88 genes that show parent-of-origin ASE bias and so we would expect that ~44 genes would show apparent paternal expression. In our data however, maternal expression is much more common than paternal expression: only nine genes are paternally expressed compared with 79 maternally expressed ( $\chi^2=55.7$ ,  $P<0.0001$ ). This is consistent with previous results showing that maternal expression is more common than paternal expression in the rodent placenta {Wang:2008jt} and suggests that apparent low library complexity is not driven by bottlenecks during library preparation.

## References

- DeVeale, B., van der Kooy, D., & Babak, T. (2012). Critical Evaluation of Imprinted Gene Expression by RNA–Seq: A New Perspective. *PLoS Genetics*, 8(3), e1002600.
- Proudhon, C., & Bourc'his, D. (2010). Identification and resolution of artifacts in the interpretation of imprinted gene expression. *Briefings in Functional Genomics*, 9(5-6), 374–384.
- R Core Team. (2008, December 1). R: A language and environment for statistical computing. Version 3.0.2. Vienna, Austria: R Foundation for Statistical Computing. Retrieved from <http://www.R-project.org>
- Wang, X., & Clark, A. G. (2014). Using next-generation RNA sequencing to identify imprinted genes. *Heredity*, 113(2), 156–166.

**THE LARGE X EFFECT, GENOMIC IMPRINTING, AND THE ARCHITECTURE OF  
EXTREME GROWTH IN DWARF HAMSTERS**

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## Abstract

Identifying the genetic basis of reproductive isolation is a primary goal in evolutionary biology. Hybrid inviability in mammals commonly manifests through extreme parent-of-origin dependent growth where reciprocal hybrids are either much larger or much smaller than the parent species. Asymmetric hybrid phenotypes imply a genetic basis that is uniparentally inherited, for example the X chromosome, mitochondria, and imprinted genes. Hybrid dwarf hamsters in the genus *Phodopus* exhibit extreme parent-of-origin growth of both placenta and embryos. Here, we use a suite of genetic and genomic experiments to test whether the X chromosome, the mitochondria, or imprinted genes are involved in parent-of-origin dependent growth in hybrid dwarf hamsters. We demonstrate a major role for the maternally inherited X chromosome, and widespread disruptions of expression of autosomal genes including imprinted genes but no influence of the mitochondria. Our data suggest that an incompatible interaction involving the maternally inherited *P. sungorus* X chromosome and a paternally inherited *P. campbelli* autosomal element results in placental and embryonic overgrowth. Overgrowth is also correlated with a greatly reduced expression of maternally-expressed imprinted genes, though any connection between expression and the X chromosome remains unclear.

## Introduction

In mammals, hybrid inviability commonly manifests through extreme parent-of-origin dependent growth where reciprocal hybrids are much larger or much smaller than the parent species. Reproductive isolation is often asymmetric in the early stages of speciation, appearing in only one of the reciprocal hybrids (Turelli and Moyle 2007). This architecture is thought to reflect the influence of genetic elements that are uniparentally inherited, such as the sex chromosomes, mitochondria, and genes with parent-specific expression (Crespi and Nosil 2013; Presgraves 2010). However, it is still unknown whether recurrent phenotypic patterns of reproductive isolation across taxa are the result of a shared genetic architecture (Butlin et al. 2012). Due to its commonality across mammals, parent-of-origin hybrid growth presents a unique opportunity to test for general patterns in the genetic architecture underlying the early stages of reproductive isolation.

Asymmetric reproductive isolation, both sterility and inviability, is often caused by the genes on X chromosome (Good, Dean, and Nachman 2008; Simon-Chazottes and Montagutelli 1990; Turelli and Orr 1995; Presgraves 2008; Presgraves 2010). This 'large-X effect' is possibly due to the differential evolutionary forces acting on the X as well as its hemizyosity in males (Tao et al. 2003; Turelli and Moyle 2007; Turelli and Orr 2000; Turelli and Orr 1995). Concerning specifically abnormal growth, the X chromosome is a likely *a priori* candidate as it is known to harbor genes which regulate the development of the placenta (Hemberger 2002). As the gateway for nutrient transfer from mother to the offspring, placental form and function is crucial for proper development of embryos. Furthermore, the X

chromosome has a complex regulatory profile such that in female embryos, one X chromosome is inactivated at random (rXCI). In the placenta however, the paternal X chromosome is silenced in what is known as imprinted X chromosome inactivation (iXCI). Placenta-specific imprinting of the X chromosome results in solely maternal expression in the placentas of both males and females. Thus an placenta-specific incompatibility involving the X chromosome is expected to affect both sexes equally, though in only one of the reciprocal hybrid types.

However, there are two other major genetic elements which could generally cause parent-of-origin effects in hybrids due to their parent-of-origin dependent inheritance: the mitochondria and imprinted genes (Vrana 2007). Due to their strictly maternal inheritance and role in regulating the energetic demands of an organism, the mitochondria are a possible genetic factor that may influence offspring growth rate. Alternatively, abnormal development may also be caused by the disruption of genomic imprinting, the parent-of-origin silencing of around 150 genes. Imprinted genes are a strong candidate for causing parent-of-origin growth because they regulate nutrient flow between the mother and her developing offspring (Vrana 2007). Disruptions in the silencing of these growth-regulating genes can result in dosage imbalances between growth promoters and repressors and cause atypical growth of offspring (Li et al. 1999; Vrana 2007; Brekke and Good 2014). However, even when the epigenetic machinery regulating imprinting is not disrupted, imprinted genes are effectively hemizygous, potentially exposing recessive incompatibilities similar to the X chromosome in males (Chakraborty 1989; Varmuza 1993).

Tests for the genetic basis of abnormal hybrid growth using deer mice and house mice have revealed some commonalities but also striking differences. In both systems the X chromosome plays an important role in placental overgrowth, but the role of imprinted autosomal genes and the importance of disrupted imprinting remains unclear (Wolf and Brandvain 2014; Zechner et al. 2004; Loschiavo et al. 2007; Duselis et al. 2005; Vrana et al. 2000). In deer mice parent-of-origin dependent overgrowth occurs when a *Peromyscus polionotus* female is crossed to a *Peromyscus maniculatus* male and males are much larger than females. Genetically this overgrowth is caused by an interaction between an X-linked region from *Peromyscus polionotus* spanning the gene *Esx1*, and an autosomal region including the imprinted gene *Peg3* (Loschiavo et al. 2007; Duselis et al. 2005). Intriguingly, *Peg3* shows loss of imprinting in large hybrids so overgrowth in deer mice appears to be caused by an interaction between the X chromosome and the imprinting of an autosomal gene (Vrana et al. 2000). Imprinted X chromosome inactivation in female hybrids is preserved, exposing this interaction in the placenta of both sexes. In the embryo however, where the X chromosome should be randomly inactivated, rXCI is skewed towards expression of the *P. maniculatus* X chromosome. As the deleterious interaction involves the *P. polionotus* X chromosome, this skewing of rXCI towards *P. maniculatus* in the embryo seems to partially mask the incompatibility in females who do not grow as extremely large as their male siblings (Vrana et al. 2000). The mitochondria do not play a role in parent-of-origin dependent growth in deer mice (Dawson et al. 1993).

In house mice, *Mus spretus* × *M. domesticus* hybrids show overgrowth and males are larger than females. Similar to deer mice, an X-linked QTL spanning the genomic location of *Esx1* is an important component causing parent-of-origin dependent overgrowth (Zechner et al. 2004; Hemberger et al. 1998). However, the incompatible interaction is between the X chromosome and an autosomal gene which is unknown (Zechner et al. 2004). Candidate gene approaches have demonstrated that imprinting is not disrupted for many genes in *Mus* hybrids (Zechner et al. 1997; Zechner et al. 2004), but this does not rule out the involvement of imprinted genes in an incompatibility. Furthermore, rXCI in the embryo is not skewed (like in deer mice) and at the tissue level females are heterozygous (Zechner et al. 2004). This functional heterozygosity may partially mask the incompatibility in female embryos and drive the sex-specific patterns of growth in the large *M. spretus* × *M. domesticus* hybrids. The influence of the mitochondria on the parent-of-origin growth in *Mus* has not been tested.

These two systems where the genetic basis of parent-of-origin growth has been dissected, both reveal a central role of the X chromosome. A general connection with genomic imprinting and imprinted loci remains elusive however, in part due to a lack of genomic scale data. Recent genome-wide scans have explored the disruptions of genomic imprinting in various taxa (hamsters (Brekke, Henry, and Good 2016), horses and donkeys (Wang, Miller, and Harman 2013), and cattle (Chen et al. 2015)) but have done so outside the context of the genetic architecture. Indeed even using transcriptomic data sets, these studies have found only mixed support for whether imprinting is disrupted in mammal hybrids. For instance, there

is no evidence for disrupted imprinting in the placenta of mules and hinnies (Wang, Miller, and Harman 2013), while cattle hybrids show much more variation with some imprinted genes expressed higher, and some lower in large hybrids (Chen et al. 2015).

Dwarf hamsters in the genus *Phodopus* (*P. sungorus* and *P. campbelli*) can hybridize and the hybrid offspring of a female *P. sungorus* (S×C) are much larger than the parents (Brekke and Good 2014). The reciprocal hybrid (c×s) are normally sized at birth, but the males show growth restriction as adults (Brekke and Good 2014). This pattern differs slightly from the house mouse and deer mouse systems as F1 hybrid hamsters do not show sex-specific growth in the large F1 hybrids. However, in dwarf hamsters, we found that a large proportion of genes with significant allele-specific expression in normal-sized F1 hybrids show expression from both alleles in large F1 hybrids, a pattern consistent with disrupted imprinting. Intriguingly, this disruption was correlated with a drastic reduction in the overall expression level of the gene. As these genes were all maternally-expressed and expected to repress growth, their low expression seems to facilitate overgrowth (Brekke, Henry, and Good 2016).

In order to uncover any generality in the genetic architecture of parent-of-origin growth in mammals, we combine genetic mapping experiments with genome-wide expression data to dissect the genetic basis of extreme parent-of-origin dependent growth in Dwarf hamsters. We first test for a role of the mitochondria using a conplastic strain of hamsters. Second, we construct the first coarse genetic map for dwarf hamsters and use it to test for the influence of any specific region of



the genome on overgrowth in hamsters. Finally we integrate our earlier findings of abnormal expression in F1 hybrids with the expression patterns of large and small backcross hybrids with the goal of identifying genes that show consistent changes in expression between large and normal placentas regardless of genetic background (F1 or backcross). We found that the X chromosome is a major factor controlling placenta growth and that the mitochondria have no influence. Furthermore, many genes, including many with significant allele-specific expression in F1 hybrids, show similar patterns of expression in large and regular backcross hybrids implying that disrupted imprinting is involved in overgrowth. Surprisingly, we find a significant sex-effect on the expression profiles in backcross individuals that was absent in F1 hybrids.

## Methods

### *Animals*

Wild-derived colonies of *P. campbelli* and *P. sungorus* were established at the University of Montana from a series of collection trips ending in the 1990s as described in (Brekke and Good 2014; Scribner and Wynne-Edwards 1994). Animals were established and maintained as an outbred colony, though with no genetic supplementation for over 20 years, inbreeding is certainly high. Animals were housed in a 14 hours light/10 hours dark daylight regimen. All experiments were done in compliance with the University of Montana Institutional Animal Care and Use Committee regulations (animal use protocol 039-13JGDBS-090413).

### *Experimental crosses*

As S×C individuals do not survive birth (Brekke and Good 2014) and c×s males are sterile (Safronova and Vasil'eva 1996; Safronova, Cherepanova, and Vasil'eva 1999; Sokolov and Vasil'eva 1993; Ishishita et al. 2015), all advanced genetic crosses had to proceed through the c×s hybrid females. To generate mitochondrial introgression lines, these F1 hybrid females were successively backcrossed to *P. sungorus* males for ten generations. As the mitochondria show strictly maternally inheritance, this crossing scheme results in hamsters that are greater than 99.9% *P. sungorus* in the nuclear genome but retain the mitochondria of *P. campbelli*. 10th-generation mitochondrial-introgression females were crossed to *P. campbelli* males to test for F1 overgrowth as this cross mimics the overgrown S×C hybrid across the nuclear genome.

Next we performed a backcross experiment by crossing F1 hybrid females to *P. campbelli* males to generate 189 individuals ([c×s]×C). These backcross hybrids mimic the overgrown F1 hybrid (paternally inherited alleles are derived from *P. campbelli*) while varying the genotype of maternally inherited alleles. This crossing scheme allowed us to test the contribution of maternally inherited genetic factors (i.e., the X chromosome and paternally imprinted autosomal genes) to placental and embryonic overgrowth, but is uninformative with respect to the contribution of paternally inherited genetic factors (i.e. the Y chromosome and maternally imprinted autosomal genes).

For both the introgression experiment and the mapping panel, females were sacrificed at late gestation and offspring placentas and embryos were harvested, weighed, and snap-frozen on dry ice. Embryos were developmentally scored as in (Brekke and Good 2014) to ensure that all offspring were in the final four days of gestation corresponding to Theiler's Stages 24-27 (see Methods in (Brekke and Good 2014; Theiler 1972)

### *Genotyping*

Genomic DNA was extracted from frozen embryos with a Machery-Nagel Nucleospin Tissue DNA extraction kit (740952). Standard kit protocols were followed with the exception that 5µl RNase-A was added to the column and incubated for 15 minutes at room temperature. Embryo sex was determined using a PCR assay of the Y-linked gene Sry as described in (Brekke, Henry, and Good 2016).

Double digest restriction-associated digest (ddRAD) libraries were generated for 189 backcross individuals (91 females and 98 males) as well as the original colony founders (14 *P. campbelli* individuals and 11 *P. sungorus* individuals) following Peterson et al. (2012) with minor modifications. We started the each library preparation with 1µg of genomic DNA (gDNA) per sample. Size selection of adapter-ligated fragments (200-500bp) was done with Agencourt AMPure XP beads (Rodrigue et al. 2010) and both size selection and PCR amplification was done prior to sample pooling to assure even representation across samples. We used the restriction enzyme *SbfI* (NEB, R3642L) as the first rare cutter and *MspI* (NEB, R0106L) as the second common cutter both with the NEB cutsmart buffer. We used a dual barcoding scheme incorporating both Illumina indexes and in-line barcodes to uniquely identify each sample (Peterson et al. 2012). The combined pools were sequenced on 50% of a Illumina HiSeq 2500 lane in rapid-run mode and then on 50% of a lane of Illumina Hiseq 2500 lane in normal mode. All samples were sequenced in each lane (Auer and Doerge 2010) and reads from both runs combined for subsequent analyses.

Multiplexed ddRAD libraries were cleaned and demultiplexed with Stacks process\_radtags (v1.20, parameters -e sbfI --renz\_2 mspI -r -c -q) (Catchen, Amores, and Hohenlohe 2011). A list of unique RADtags (unique sequences drawn from the pool of sequenced reads) from both first and second reads was generated using ustacks (-H -r -d) with two female founders, one of each species. Then RADtag reference libraries were generated using cstacks (-n 4). By using the genotypes of the animals which founded our colony, we can restrict the analysis to only sites that

are fixed between the species. Reads from all the founders were aligned to the RADtag reference library with bwa mem (v0.7.9a) (H. Li and Durbin 2009) and single-nucleotide variants (SNVs) were called with the GATK HaplotypeCaller (v3.1-1, -stand\_call\_conf 30) (Van der Auwera et al. 2013; McKenna et al. 2010; DePristo et al. 2011). All SNVs that were polymorphic within a species in our colony were filtered out using GATK selectVariants (v3.1-1) (Van der Auwera et al. 2013; McKenna et al. 2010; DePristo et al. 2011) resulting in over 2,000 SNVs fixed between the species in our colony (due to inbreeding in the lab, these SNVs may or may not be completely fixed between these species in the wild). Backcross individuals were genotyped at these fixed SNVs using GATK UnifiedGenotyper (v3.1-1, -stand\_call\_conf 30) (Van der Auwera et al. 2013; McKenna et al. 2010; DePristo et al. 2011).

### *Quantitative genetic analysis*

We first constructed a genetic map using the set of fixed SNVs identified between the strains of *P. campbelli* and *P. sungorus* and the program R/qtl (Broman 2012). X-linked RADtags were manually identified as those markers that were either homozygous *P. campbelli* or heterozygous in backcross females and always homozygous in backcross males for either *P. campbelli* or *P. sungorus*. To build the map, we first removed two backcross individuals who had low sequencing coverage and then dropped all autosomal markers that were genotyped in less than 177 individuals. We formed linkage groups and ordered the markers on each linkage group with the `ripple()`, `compareorder()`, and `switch.order()` functions until each

linkage group was as short as possible. Then we sequentially dropped each marker to see if the likelihood of the map improved. Once all poor quality markers were removed, we repeated the `ripple()`, `compareorder()`, and `switch.order()` functions until the likelihood was maximized.

Using R/qtl we tested for quantitative trait loci (QTL) associated with the variation in embryo and placenta weight in our backcross mapping panel (Broman and Sen 2009; Broman 2012). We first estimated single QTL across the genome for both embryo weight and placenta weight. We used the extended Haley-Knott method and the imputation method for estimating QTL (Haley and Knott 1992; Feenstra, Skovgaard, and Broman 2006). Next, we incorporated sex as a covariate and re-estimated the QTL for both embryo weight and placenta weight. Finally, we used the QTL identified in the first two analyses as additive cofactors and re-scanned for additional QTL for both embryo and placenta weight that are contingent on the presence of the earlier identified QTL (Broman and Sen 2009). To identify a significance threshold for QTL, we used a permutation test with 10,000 permutations. QTL intervals were established with 95% Bayesian confidence interval (Broman and Sen 2009).

### *Gene expression analyses*

To complement the genetic mapping experiments, we chose 24 backcross placentas for genome-wide expression analysis using RNAseq. All of these individuals were included in the RAD panel and chosen based on placenta size and gender: we sequenced six males and six females with large placentas ( $0.232 \pm 0.010$ g) and six

males and six females with normal placentas ( $0.140 \pm 0.008\text{g}$ ). These weights were chosen to reflect overgrown S×C hybrid placentas ( $0.420 \pm 0.134\text{g}$ ) and parental placentas ( $0.127 \pm 0.300\text{g}$ ) as closely as possible (Brekke and Good 2014). RNA was extracted from whole frozen placenta with a E.Z.N.A. Total RNA Kit I (R6834-50). A DNase digestion was performed as per the kit protocol. All RNA samples were checked for quality and concentration on the bioanalyzer and all samples used had RNA integrity numbers greater than 8.0.

RNAseq libraries were constructed with the Agilent Sure-Select Strand-Specific RNAseq Kit (G9691B) as per the manufacturers recommendations. Libraries were built with 2 micrograms of input RNA, amplified with 14 cycles of PCR, and pooled based on a Kappa Quantification Kit (KK4824). The pooled libraries were sequenced with two lanes of Illumina HiSeq2500 100bp single-end sequencing.

RNAseq libraries were processed as described in (Brekke, Henry, and Good 2016). In short, Illumina adapters were trimmed off reads with cutadapt -O 5 -e 0.1 (Martin 2011) and quality trimmed with trimmomatic SE -phred 33 LEADING:5 SLIDINGWINDOW:4:15 HEADCROP:13 (Bolger, Lohse, and Usadel 2014). Reads were aligned to the transcriptomes generated by (Brekke, Henry, and Good 2016). To evaluate differential expression, we created a table of counts at the gene level using featureCounts (v1.4.2) which counted fragments (-p) and discarded those that have too long an insert (-P) or are chimeric (-C) or have a mapping quality (-Q) below 20 (Liao, Smyth, and Shi 2014). This table of counts was normalized with the TMM method (Robinson and Oshlack 2010) and analyzed with the generalized

linear model approach implemented in edgeR(Robinson, McCarthy, and Smyth 2010; McCarthy, Chen, and Smyth 2012).

We tested for enrichment of gene ontology in groups of differentially expressed genes using DAVID (Huang, Sherman, and Lempicki 2009a; Huang, Sherman, and Lempicki 2009b). DAVID analyses were run using the *Phodopus* placental transcriptome as a background and the functional annotation clustering was run for the gene ontology term categories ‘molecular function’ and ‘biological process’.



## Results

### *Mitochondria have no effect on F1 hybrid overgrowth*

Due to their strict maternal inheritance, the mitochondria are a possible cause of parent-of-origin dependent growth. Normally, the offspring of a *P. sungorus* female crossed to a *P. campbelli* male (S×C) are overgrown. Here we introgressed *P. campbelli* mitochondria into the genetic background of *P. sungorus* through ten generations of backcrossing. Using these conplastic *P. sungorus* females in a cross with *P. campbelli* males ( $S^{mtC} \times C$ ) eliminates the possibility for a negative interaction between *P. sungorus* mitochondria and the *P. campbelli* nuclear genome. If an interaction between the mitochondria and the nuclear genome is the cause of overgrowth in F1 hybrids, then  $S^{mtC} \times C$  hybrids will not show the overgrowth phenotype. Alternatively, if the mitochondria have little or no effect on growth, then  $S^{mtC} \times C$  hybrids will be of similar size to the overgrown S×C hybrids. In fact,  $S^{mtC} \times C$  placentas are extremely large and statistically similar to S×C hybrids (Figure 1; data for *P. campbelli*, c×s, S×C, and *P. sungorus* from (Brekke and Good 2014);  $F_{4,213} = 106$ ,  $P < 0.001$ , ANOVA, Tukey test reveals that S×C and  $S^{mtC} \times C$  are similar to each other but different from all other cross types). Ostensibly, the mitochondria rescue embryo size as only S×C hybrids are large in the final four days of gestation ( $F_{4,210} = 13.9$ ,  $P < 0.001$ , ANOVA, Tukey test identifies S×C as the only outlier). However, as opposed to the placenta, which gains most of its mass early in development, the embryo grows exponentially through gestation and gains most of its mass in the final days. Thus, using only embryos from the final two days of gestation reveal that

the mitochondrial introgression fails to rescue embryo size ( $F_{4,127} = 13.2$ ,  $P < 0.001$ , ANOVA, Tukey test reveals that  $S^{mtC} \times C$  and  $S \times C$  are similar to the exclusion of all other cross types).

### *The Phodopus genetic map*

While there is no physical map for dwarf hamsters, karyotypes have been reported and describe five large chromosomes, four medium chromosomes, and five small chromosomes with the X falling in the 'medium' category (Gamperl, Vistorin, and Rosenkranz 1977; Haaf, Weis, and Schmid 1987; Van Hoosier 1987; Romanenko et al. 2007). Recapitulating the karyotype, our genetic map grouped into 13 autosomal linkage groups and the X chromosome. It includes 1,215 RAD markers and spans 1,231.7 cM (Figure 2 and Supplementary Table 1). The relative sizes of the linkage groups in the genetic map reflect the described karyotype closely but not perfectly. The genetic map reveals three large ( $>125\text{cM}$ ), four medium ( $>80\text{cM}$ ), and seven small ( $<80\text{ cM}$ ) linkage groups with the X chromosome being the shortest of all. While less robust than a comparison between genetic and physical maps, this pattern suggests some that genomic rearrangements have occurred during the divergence of *P. campbelli* and *P. sungorus* as such rearrangements are known to suppress recombination and shorten the length of a genetic map (Livingstone, Churchill, and Jahn 2000). Indeed, earlier claims that the X chromosome has experienced rearrangements (Ross 1995; Vorontsov 1967) are supported by the short size of the X chromosome in our map.

### *The genetic architecture of extreme hybrid overgrowth*

Two phenotypes, placental weight and embryo weight were collected for all backcross animals (Figure 3A, B; data for parental and S×C weights from (Brekke and Good 2014)). Placenta and embryo weights are correlated in males, though with low  $r^2$  (Figure 3C,  $r^2 = 0.251$ ,  $F_{1,93} = 32.5$ ,  $P < 0.0001$ , ANOVA), but no correlation exists in females (Figure 3C,  $r^2 = 0.008$ ,  $F_{1,89} = 1.69$ ,  $P > 0.05$ , ANOVA).

There is a very strong association between the X chromosome and placental weight (Figure 4A). This QTL peaks at 31.1cM and has a 95% bayesian confidence interval between 29.6cM and 32.6cM, though likely due to the suspected rearrangement, the entire X chromosome exceeds the  $P = 0.01$  significance threshold. The *P. sungorus* X chromosome is thus a major factor that causes placentas to be large. It appears fully dominant and increases placental size by ~60% (Figure 4A inset,  $F_{1,179} = 178.4$ ,  $P < 0.0001$ , ANOVA). Next, in hopes of identifying additional QTL, we repeated the scan using first sex and then the X-linked QTL as a cofactor. However, no new QTL were uncovered in either of these subsequent scans.

A basic scan for QTL for embryo weight found no LOD peaks crossing the  $P = 0.5$  significance threshold (data not shown). However, When accounting for sex, there is a QTL for embryo weight on linkage group 5 that is significant at the  $P = 0.05$  threshold (Figure 4B). This QTL is centered at 63.0cM and the 95% bayesian confidence interval spans from 50.0cM to 70.7cM. This QTL influences embryo weight in females but not males (Figure 4B inset,  $F_{3,176} = 16.5$ ,  $P < 0.0001$ , ANOVA). The presence of the *P. sungorus* allele significantly increases female weight by ~20%

(Tukey test  $P = 0.003$ ), while a similar magnitude decrease in average male weight is nonsignificant (Tukey test  $P = 0.107$ ). No additional QTL were uncovered when accounting using the linkage group 5 QTL as a cofactor.

#### *Disrupted placental expression associated with extreme growth*

Our first goal was to identify genes with differential expression between large and normal backcross placentas. There were 498 genes with differential expression between the large and normal backcross size-classes, 454 autosomal and 44 X-linked, and these were enriched for gene ontologies involved in vascular development (Supplemental Table 2A). 329 genes had lower expression in large than normal backcrosses and were enriched for gene ontology categories including angiogenesis, cell migration, and the regulation of cell locomotion (Supplemental Table 2B). 169 genes had higher expression in large backcrosses and had no significant gene ontology enrichment categories.

Our next goal was to identify any sets of genes with consistent expression patterns in backcrosses and F1 hybrids. Genes whose expression is always high or low in large placentas regardless of the genetic background are those whose expression is fundamentally linked with abnormal growth. We incorporated the expression data from backcross placentas with our previously generated and analyzed expression profiles from the placentas of *P. campbelli*, *P. sungorus*, and the reciprocal F1 hybrids (c×s and S×C) (Brekke, Henry, and Good 2016). Earlier, we described 1,471 genes that fall outside the parental range in S×C hybrids (Brekke, Henry, and Good 2016). Presumably, some of this set are genes whose abnormal

expression causes placental overgrowth, some are genes whose expression is a result of abnormal growth, and some are genes whose expression is not related to abnormal growth and just happen to be mis-expressed in those hybrid individuals. In combining these two datasets, 165 genes (160A, 10X) emerged whose expression patterns were consistently correlated with overgrowth in all genetic backgrounds (Figure 5). 30 genes (28A, 2X) had consistently higher expression in large placentas, and 135 (127A, 8X) had consistently lower expression. These expression profiles group the backcross hybrids into two major clusters by size and to a lesser extent by sex, though a few of the normal individuals cluster with the large males (Figure 5). This set included many genes known to play a role in embryonic development such as *Mash2* (Oh-McGinnis, Bogutz, and Lefebvre 2011; Guillemot et al. 1995) and the hox genes *Hoxa11*, *Hoxd9*, and *Hoxd10*. Imprinted genes, such as the known tumor suppressors *Tfpi2* (Takada et al. 2010), *Osbp15* (Higashimoto et al. 2006), and *Wt1* (Rauscher 1993), are strongly over-represented in genes with low expression in large backcrosses; 31 of which show significant allele-specific expression in F1 hybrids (Brekke, Henry, and Good 2016). Imprinted genes represent approximately 0.97% of the hamster placental transcriptome (88 out of 9,041 genes show allele-specific expression in hamsters (Brekke, Henry, and Good 2016)) but comprise 19% of the genes that show consistent abnormal expression between large and small hybrids (31 out of 165,  $P < 0.0001$ , Fisher's Exact Test). All 31 of these genes were identified as potentially maternally-expressed imprinted genes in F1 hybrids. 26 of them gained imprinting in S×C hybrids concomitant with significantly lower expression. As maternally expressed genes tend to repress growth, their low

expression in large placentas is consistent with their expected functional role and associated overgrowth phenotype.

Despite the lack of sex-specific effects in the phenotype or transcriptomes of F1 hybrids, we did identify a sex-specific QTL for embryo growth which motivates a close inspection of sex-specific patterns on gene expression in the backcross placentas. In order to uncover sex-specific patterns of autosomal gene expression we modeled expression on both placenta size and sex. As suggested by the cluster analysis, few differences exist between sexes with normal placentas (Figure 5). In fact *Xist*, *Kdm5d*, and an autosomal immunoglobulin, *Sema3c* are the only differentially expressed genes between the normal males and normal females. Intriguingly, 34 genes (31A, 2X, 1Y) were differentially expressed between large females and large males, none of which were potential imprinted genes in F1 hybrids. We compared each sex of the large size class to the same sex of the normal size classes and found that large females typically show more extreme differential expression than large males. Large females had 723 differentially expressed genes (688A, 35X) compared to the normal females while large males had only 2 (1A, 1X) compared with small males. This represents a significant sex-specific effect where large backcross females show more extreme expression than large backcross males (Figure 6). The dearth of significant male-specific differences is somewhat misleading as large male expression normally trends in the same direction as large females, just not as severely. Nonetheless, this contrasts sharply with the pattern in F1 hybrid placenta where there are no major differences between the sexes of each hybrid type.

## Discussion

The X chromosome plays a major role in the genetics of hybrid inviability in hamsters, specifically regarding parent-of-origin overgrowth. Furthermore, placental overgrowth is associated with a widespread misregulation of autosomal genes across a variety of genetic backgrounds. Sex-specific effects on expression are apparent in the placentas of large backcross hybrids where females show more extreme expression than males. Finally, despite their maternal inheritance and role in regulating energy use, the mitochondria do not influence placental size in dwarf hamsters.

### *The large X effect and parent-of-origin hybrid growth*

The X chromosome is a major component responsible for parent-of-origin dependent overgrowth in hamster hybrids. Inheriting a maternally-derived *P. sungorus* X chromosome increases placenta weight by approximately 60% (Figure 3b). The *P. sungorus* X chromosome likely interacts with a *P. campbelli* autosomal factor. Unfortunately, our mapping panel is unable to identify the interacting partner because of the nature of the backcross-crossing scheme, which only varies the maternal genome.

The importance of the X chromosome in causing abnormal growth in dwarf hamsters is reflected in deer mice and house mice. In all three systems, an interaction with the X chromosome is fundamental for causing placental overgrowth in hybrids (Vrana et al. 2000; Duselis et al. 2005; Loschiavo et al. 2007; Zechner et

al. 1996; Zechner et al. 1997; Zechner et al. 2004; Hemberger et al. 1999; Hemberger et al. 2001). In general, X-linked QTL are expected to associate with sex-specific phenotypes as males are hemizygous while females have two X chromosomes. All taxa examined display some pattern of sex-specific phenotype, in deer mice and house mice F1 size is slightly dimorphic *in utero*, while in hamsters size differences only become pronounced in adults (Brekke and Good 2014). In this regard, dwarf hamsters are somewhat unusual as there are no sex effects on placenta or embryo weight in F1 or backcross hybrids. This is likely due to imprinted X chromosome inactivation that occurs in the female placenta. Females silence their paternally derived X chromosome in the placenta and only express the maternal copy (Wake, Takagi, and Sasaki 1976). As males are hemizygous and only have a maternal X, both sexes are expected to have solely maternal expression from the X chromosome in the placenta. Sex-specific effects in the placentas of deer mice and house mice could theoretically be due to a disruption of iXCI, but empirically the X chromosome appears properly imprinted in the placenta of both of these systems (Hemberger et al. 2001; Vrana et al. 2000) and dwarf hamsters as well (Brekke, Henry, and Good 2016).

To explain the sex effects in house mice, Hemberger (2001) suggest that the Y chromosome may be involved. Alternatively, expression of the X chromosome in the embryo may influence placental weight. In embryonic tissue females randomly inactivate one X chromosome and so on the tissue-level they are functionally heterozygous. If embryonic expression acts as a buffer for placental expression, it is possible that random X chromosome inactivation in embryos may facilitate sex-



specific effects in placentas by partially masking any recessive incompatibilities in females. A slight variation on this is what seems to cause sex-specific growth in deer mice. In deer mouse hybrids an interaction involving the *Peromyscus polionotus* X chromosome causes overgrowth, but rXCI in the embryo is actually skewed towards the *P. maniculatus* X chromosome, effectively masking the incompatibility in females but not males (Vrana et al. 2000). As a final alternative, there are genes with sex-specific expression that are not X-linked. Indeed, despite the apparent lack of phenotypic sex-effects in F1 hybrid hamsters, we have identified a sex-specific QTL for embryo weight in backcrosses. Surprisingly, it is not on the X, but on linkage group 5 where maternally inherited *P. sungorus* alleles increase female embryo weight by approximately 20% (Figure 3d). This sort of autosomal sex-effect QTL may be more common than appreciated and drive sexual size dimorphism *in utero*.

#### *Disrupted gene expression and the role of genomic imprinting*

Since its discovery, genomic imprinting has been closely associated with abnormal growth. Multiple lines of evidence link abnormal growth with imprinted genes in both the placenta and embryo, and at various ages from conception through adulthood. Imprinting was first identified when attempts at cloning mice from two egg or two sperm pronuclei resulted in failed development due to abnormal growth of the embryo and placenta (Barton, Adams, and Norris 1985; Surani and Barton 1983). Since then, various types of mutations in imprinted genes or their epigenetic regulation have been linked to growth-related human diseases such as Angelman syndrome, Beckwith-Wiedemann syndrome, Silver-Russel syndrome, and Prader-

Willi syndrome (Butler 2009), as well as Wilms tumors and many other cancers (Lim and Maher 2010). There is strong evidence that imprinted genes directly regulate growth of the placenta and embryo in mouse models (Constancia et al. 2002) and disruptions in the epigenetic regulation of imprinted genes has been linked to growth and development in mammal F1 hybrids in cattle (Chen et al. 2015), and deer mice (Vrana et al. 1998; Vrana et al. 2000). Given this breadth of evidence, we evaluated the expression level of imprinted genes in hamster hybrids and found that imprinted genes, and particularly maternally expressed genes, tend to show greatly reduced expression along with the gain of expression of the normally silenced allele (Brekke, Henry, and Good 2016). This pattern, which we term gain-of-imprinting as it is consistent with the silencing of the normally active allele, occurs in maternally expressed growth repressors in S×C F1 hybrids and correlates well with their large size (Brekke, Henry, and Good 2016).

Despite the mass of evidence linking imprinting with abnormal growth, very few studies have evaluated the role of imprinting in the context of genetic architecture. Expression profiles in backcrosses tend to have much more variation than that in F1 hybrids due to the stochasticity inherent in segregation; fortunately transcriptome-wide approaches are often powerful enough to extract a signal from the noise (Dion-Cote et al. 2014). The high amount of noise in backcrosses is apparent in our study where some normal individuals cluster with the large individuals (Figure 5), but we are still able to identify striking signatures of differential expression that correlate with size. Indeed, we identified nearly 500 genes with differential expression between the placentas of large and normal-sized

backcross hybrids. The abnormal expression level of these genes may cause abnormal overgrowth, be a consequence of disrupted growth, or their misregulation may be completely coincidental to the size of the placenta in which they are expressed. Similarly, there is a set of 1,471 genes that show disrupted expression in large S×C F1 hybrids any of which may be the cause of-, caused by-, or coincident to the size of the hybrid. Intriguing are the 165 genes that overlap between these two sets as they represent the core genes whose expression is strongly correlated with overgrowth in 13 unique genomic backgrounds (the F1 and 12 independently segregated backcrosses). While this assay can not distinguish between genes that may actually cause overgrowth versus the ones whose disrupted expression is the result of overgrowth, it does significantly restrict the list to those tightly linked to disrupted growth given the huge variety of genomic backgrounds. The majority of these have reduced expression in the large placentas (135 of 165) and are highly enriched for maternally-expressed imprinted genes (31 of 165). As a class, maternally expressed genes tend to restrict overall offspring growth and so the repressed expression of these genes acts as a release of inhibition on growth in large F1 and backcross placentas. The striking agreement in expression level, functional role, and placenta size between such different genetic backgrounds as F1s, heterozygous at all loci, and 12 backcrosses, each with their own unique patterns of segregation, strongly suggests that the expression of these genes is an important facet of abnormal placental growth. The huge enrichment of imprinted genes in this set attests to how tightly linked imprinting is with placental overgrowth.

Along with reduced expression, many maternally-expressed genes show bi-allelic expression in large F1 hybrids. We are yet unable to evaluate allele-specific expression in backcross offspring as we do not have access to the genotype at every locus for each individual which is necessary for such an analysis. To circumvent this problem, we have designed a custom exon capture which will allow us to genotype each individual at the imprinted genes. This analysis will reveal whether imprinting is actually disrupted at these genes or whether they have simply experienced a repression of overall expression.

Sex effects for expression were unexpected in the backcross as they do not occur in F1 hybrids, nor is there any clear sexual size dimorphism in backcrosses. Nonetheless large female hybrids tend to show more extreme expression differences than large males (Figure 6). Sex-specific differences in expression may represent a disruption of gene expression that is uncovered only in later hybrids (i.e.: backcrosses) as recessive-recessive interactions are masked in F1s. It may be that the X chromosome is involved, but testing how likely that is requires first determining whether imprinted X chromosome inactivation is properly maintained in backcrosses as it is in F1s.

#### *Architecture of growth-related inviability*

Our data suggests a correlation between the genotype of the X chromosome and the expression patterns of the autosomes including the imprinted genes. Abnormal autosomal expression and disrupted imprinting may represent a downstream consequence of incompatibilities associated with the X or more intriguingly, a *P*.

*sungorus* element from the X may be trans-acting factor incompatible with the autosomes which disrupts the gene networks regulating embryonic growth. A large scale expression QTL study and additional genetic crosses will help to resolve these issues.

A second question that remains is whether paternally inherited factors may play a role in overgrowth. Our mapping panel was limited to only identifying maternally inherited elements that influence growth due to our use of a backcross- rather than an F2- crossing scheme. This decision was necessitated by the sterility of hybrid males (Safronova and Vasil'eva 1996; Safronova, Cherepanova, and Vasil'eva 1999; Ishishita et al. 2015), but it is likely that paternally inherited elements are also important and in order to identify those, a more complicated crossing scheme is necessary. One option would be to use advanced hybrids rather than F1s to generate the mapping panel as it is possible that male fertility may be restored in later generation backcrosses. Indeed similar approaches has been used in deer mice (Vrana et al. 2000) and house mice (Zechner et al. 2004). A crossing scheme where the maternal X derives from *P. sungorus* while the paternal input is variable would be necessary to address this question.

Finally, while it is often assumed that the placenta size directly influences the embryo size, our data suggest that the connection between placental and embryonic size is weak in hamsters (Figure 3C) and similar findings have been reported in house mice (Kurz et al. 1999). In apparent contrast, knocking out the expression of the imprinted gene *Igf2* results in placental undergrowth that precedes embryonic growth restriction by only a few days (Constancia et al. 2002). The placenta is

fundamentally important for proper mammalian development and yet it is one of the most rapidly evolving organ in mammals both genetically (Chuong, Tong, and Hoekstra 2010), and in terms of gross morphology (Leiser and Kaufmann 1994). Furthermore, hybrid placental dysplasia seems to manifest earlier in divergence than embryonic or adult growth phenotypes (Brekke and Good 2014). Embryos are clearly able to withstand variation in placenta form and function throughout their development. To reconcile both the developmental necessity of the placenta with its rapid evolution, and the apparent disconnect between embryo and placental sizes, we suggest that embryos may be more adept at buffering the effects of the placenta than previously thought, and that the effect of placental size on embryonic growth may occur only once the dysgenesis of the placenta crosses a threshold.

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## Figure Legends

Figure 1. Mitochondria have no effect on placenta size. If an interaction involving the *P. sungorus* mitochondria causes overgrowth, introgressing *P. campbelli* mitochondria onto a *P. sungorus* nuclear background ( $S^{mtC}$ ) should rescue the phenotype. Instead the placentas from offspring of  $S^{mtC}$  females crossed back to *P. campbelli* males ( $S^{mtC} \times C$ ) are indistinguishable in size from placentas in  $S \times C$  hybrids ( $F_{4,213} = 106$ ,  $P < 0.001$ , ANOVA). Data for *P. campbelli*,  $c \times s$ ,  $S \times C$ , and *P. sungorus* from (Brekke, 2014). Statistically significant groups 'A' and 'B' were assigned with a Tukey HSD test.

Figure 2. Genetic map of *Phodopus*. This map includes 1,215 RAD markers and spans 1,213.7 cM across 13 autosomal linkage groups and the X chromosome. Linkage groups are numbered by in decreasing order based on the number of markers they contain. The X chromosome shows high marker density and is relatively much smaller than described from karyotype studeis, suggesting rearrangements have occurred between *P. campbelli* and *P. sungorus*. Further data on marker sequences and exact locations in centiMorgans can be found in Supplementary Table 1.

Figure 3. Placenta and embryo weights. Backcross placenta (A) and embryo (B) weights show high variance and span the range from the average parental size (gray) to average  $S \times C$  (black) size. Blue denotes backcross males and yellow denotes backcross females. (C) The correlation between embryo and placenta weights is



significant although weak in males ( $r^2 = 0.251$ ,  $F_{1,93} = 32.5$ ,  $P < 0.0001$ , ANOVA), but not significant in females ( $r^2 = 0.008$ ,  $F_{1,89} = 1.69$ ,  $P > 0.05$ , ANOVA).

Figure 4. QTL for placenta and embryo weight. Significance thresholds are denoted by solid ( $P = 0.01$ ) and dashed ( $P = 0.05$ ) horizontal lines. (A) A QTL for placenta weight is found on the X chromosome and centered on marker 3628\_61 located at 31.1cM with a 95% bayesian confidence interval between 29.6cM and 32.6cM. Possibly due to a suspected rearrangement, the entire X chromosome exceeds the  $P = 0.01$  significance threshold. The *P. sungorus* X chromosome increases placenta weight by ~60% (inset,  $F_{1,179} = 178.4$ ,  $P < 0.0001$ , lettered groups assigned by a Tukey test). Placenta weights are plotted depending on the genotype at marker 3628\_61 which is found at the peak of the QTL. Genotypes are denoted with the maternally derived allele first followed by the paternally derived allele. Note that in the placenta only the maternal X chromosome is expressed in hamsters. (B) When sex is taken as a cofactor, there is a QTL for embryo weight that is significant at the  $P = 0.05$  significance threshold which centers on marker 5812\_32 located at 63.0cM with a 95% bayesian confidence interval from 50.0cM to 70.7cM. The presence of a *P. sungorus* allele at marker 5812\_32 on linkage group 4 causes female embryos to be ~20% larger, though a similar decrease in male size is not significant (inset,  $F_{3,176} = 16.5$ ,  $P < 0.0001$ , lettered groups assigned by a Tukey test).

Figure 5. Gene expression and clustering of backcross placentas. Plotted are the expression level of 165 genes in backcross placentas. These 165 genes have consistent expression profiles between large and normal placentas regardless of genetic background (F1 or backcross). Clustering placentas based on similar expression of these genes reveals two major clusters, generally representing normal and large placentas, though with some noise. A second cluster between large females and large males is also apparent.

Figure 6. Sex-specific effects on expression. The absolute value of the log fold-change between large and normal placentas within each sex. Females tend to have more extreme expression differentials than males. This is true for all genes ( $n=12,845$ , t test,  $P < 0.0001$ ), the subset of all genes which are differentially expressed between large and normal placentas ( $n=498$ , t test,  $P < 0.0001$ ), the subset of which have consistent expression in F1 and backcross placentas ( $n=165$ , t test,  $P < 0.0001$ ), and the subset of which are potential imprinted genes identified in (Brekke, Henry, and Good 2016) ( $n=31$ , t test,  $P < 0.0001$ ). Boxes represent the median, 1<sup>st</sup> and 3<sup>rd</sup> quartiles, and 95% confidence intervals.

Supplemental Table 1. A full description of all RAD markers including their ID, the linkage group they are found on, the position in centiMorgans on that linkage group, the base position of the SNV between *P. campbelli* and *P. sungorus*, the alleles for *P. campbelli* and *P. sungorus*, and the sequence of the marker which always begin

with TGCAGG, the restriction enzyme cut site of SbfI (CC\_TGCA^GG). SNVs in the sequence are denoted with standard IUPAC ambiguity codes.

## Literature Cited

- Auer, P L, and R W Doerge. 2010. "Statistical Design and Analysis of RNA Sequencing Data." *Genetics* 185 (2): 405–16. doi:10.1534/genetics.110.114983.
- Barton, S C, C A Adams, and M L Norris. 1985. "Development of Gynogenetic and Parthenogenetic Inner Cell Mass and Trophectoderm Tissues in Reconstituted Blastocysts in the Mouse." *Journal of Embryology and Experimental Morphology* 90: 267-285.
- Bolger, Anthony M, Marc Lohse, and Bjoern Usadel. 2014. "Trimmomatic: a Flexible Trimmer for Illumina Sequence Data." *Bioinformatics* 30 (15): 2114-2120. Oxford University Press: 2114–20. doi:10.1093/bioinformatics/btu170.
- Brekke, Thomas D, and Jeffrey M Good. 2014. "Parent-of-Origin Growth Effects and the Evolution of Hybrid Inviability in Dwarf Hamsters." *Evolution* 68 (11): 3134–48. doi:10.1111/evo.12500.
- Brekke, Thomas D, Lindy Henry, and Jeffrey M Good. 2016. "Disrupted Placental Expression, Genomic Imprinting, and Speciation in Dwarf Hamsters." *In Prep*. Chapter 2.
- Broman, Karl W. 2012. "Genetic Map Construction with R/Qtl." *University of Wisconsin-Madison Department of Biostatistics & Medical Informatics Technical Report* 1–41.
- Broman, Karl W, and Saunak Sen. 2009. *A Guide to QTL Mapping with R/Qtl*. Springer.

- Butler, Merlin G. 2009. "Genomic Imprinting Disorders in Humans: a Mini-Review." *Journal of Assisted Reproduction and Genetics* 26 (9-10): 477–86. doi:10.1007/s10815-009-9353-3.
- Butlin, Roger, Allan DeBelle, Claudius Kerth, Rhonda R Snook, Leo W Beukeboom, Ruth F Castillo Cajas, Wenwen Diao, et al. 2012. "What Do We Need to Know About Speciation?" *Trends in Ecology & Evolution* 27 (1): 27–39. doi:10.1016/j.tree.2011.09.002.
- Catchen, J M, A Amores, and P Hohenlohe. 2011. "Stacks: Building and Genotyping Loci De Novo From Short-Read Sequences." *G3: Genes/ Genomes/ Genetics* 1 (3): 171-182. doi:10.1534/g3.111.000240/-/DC1.
- Chakraborty, R. 1989. "Can Molecular Imprinting Explain Heterozygote Deficiency and Hybrid Vigor?" *Genetics* 122 (3): 713–17. doi:10.2307/2469503?ref=no-x-route:d07c394c5181b00fa6da1a3ec7754efa.
- Chen, Zhiyuan, Darren E Hagen, Christine G Elisk, Tieming Ji, Collin James Morris, Laura Emily Moon, and Rocío Melissa Rivera. 2015. "Characterization of Global Loss of Imprinting in Fetal Overgrowth Syndrome Induced by Assisted Reproduction." *Proceedings of the National Academy of Sciences* 112 (15): 4618–23. doi:10.1073/pnas.1422088112.
- Chuong, E B, W Tong, and H E Hoekstra. 2010. "Maternal-Fetal Conflict: Rapidly Evolving Proteins in the Rodent Placenta." *Molecular Biology and Evolution* 27 (6): 1221–25. doi:10.1093/molbev/msq034.

- Constancia, M, M C Hemberger, J Hughes, W Dean, Anne C Ferguson-Smith, Reinald H Fundele, F Stewart, G Kelsey, A Fowden, and C Sibley. 2002. "Placental-Specific IGF-II Is a Major Modulator of Placental and Fetal Growth." *Nature* 417 (6892): 945–48.
- Crespi, Bernard J, and Patrik Nosil. 2013. "Conflictual Speciation: Species Formation via Genomic Conflict." *Trends in Ecology & Evolution* 28 (1): 48–57.  
doi:10.1016/j.tree.2012.08.015.
- Dawson, W D, M N Sagedy, L En-yu, D H Kass, and J P Crossland. 1993. "Growth Regulation in *Peromyscus* Species Hybrids: a Test for Mitochondrial-Nuclear Genomic Interaction." *Growth, Development, and Aging : GDA* 57 (2): 121–33.
- DePristo, Mark A, Eric Banks, Ryan Poplin, Kiran V Garimella, Jared R Maguire, Christopher Hartl, Anthony A Philippakis, et al. 2011. "A Framework for Variation Discovery and Genotyping Using Next-Generation DNA Sequencing Data." *Nature Genetics* 43 (5): 491–98. doi:10.1038/ng.806.
- Dion-Cote, A M, S Renaut, E Normandeau, and L Bernatchez. 2014. "RNA-Seq Reveals Transcriptomic Shock Involving Transposable Elements Reactivation in Hybrids of Young Lake Whitefish Species." *Molecular Biology and Evolution* 31 (5): 1188–99.  
doi:10.1093/molbev/msu069.
- Duselis, Amanda R, Christopher D Wiley, Michael J O'Neill, and Paul B Vrana. 2005. "Genetic Evidence for a Maternal Effect Locus Controlling Genomic Imprinting and Growth." *Genesis* 43 (4): 155–65. doi:10.1002/gene.20166.

- Feenstra, Bjarke, Ib M Skovgaard, and Karl W Broman. 2006. "Mapping Quantitative Trait Loci by an Extension of the Haley-Knott Regression Method Using Estimating Equations." *Genetics* 173 (4): 2269–82. doi:10.1534/genetics.106.058537.
- Gamperl, Roswitha, Gerda Vistorin, and W Rosenkranz. 1977. "New Observations on the Karyotype of the Djungarian Hamster, *Phodopus Sungorus*." *Experientia* 33 (8): 1020–21. doi:10.1007/BF01945943.
- Good, Jeffrey M, Matthew D Dean, and Michael W Nachman. 2008. "A Complex Genetic Basis to X-Linked Hybrid Male Sterility Between Two Species of House Mice." *Genetics* 179 (4): 2213–28. doi:10.1534/genetics.107.085340.
- Guillemot, F, T Caspary, S M Tilghman, N G Copeland, D J Gilbert, N A Jenkins, D J Anderson, A L Joyner, J Rossant, and Andras Nagy. 1995. "Genomic Imprinting of *Mash2*, a Mouse Gene Required for Trophoblast Development." *Nature Genetics* 9 (3): 235–42. doi:10.1038/ng0395-235.
- Haaf, T, H Weis, and M Schmid. 1987. A Comparative Cytogenetic Study on the Mitotic and Meiotic Chromosomes in Hamster Species of the Genus *Phodopus* (Rodentia, Cricetinae). *Zeitschrift für Säugetierkunde* 52: 281-290 . doi:10.1159/000132316.
- Haley, C S, and S A Knott. 1992. "A Simple Regression Method for Mapping Quantitative Trait Loci in Line Crosses Using Flanking Markers." *Heredity* 69 (4): 315–24. doi:10.1038/hdy.1992.131.
- Hemberger, M C. 2002. "The Role of the X Chromosome in Mammalian Extra Embryonic

Development.” *Cytogenetic and Genome Research* 99 (1-4): 210–17.

doi:10.1159/000071595.

Hemberger, M C, H Kurz, Annie Orth, Sabine Otto, A Lüttges, Rosemary W Elliott, Andras Nagy, et al. 2001. “Genetic and Developmental Analysis of X-Inactivation in Interspecific Hybrid Mice Suggests a Role for the Y Chromosome in Placental Dysplasia.” *Genetics* 157 (1): 341–48.

Hemberger, M C, R S Pearsall, U Zechner, Annie Orth, Sabine Otto, F Rüschemdorf, Reinald H Fundele, and Rosemary W Elliott. 1999. “Genetic Dissection of X-Linked Interspecific Hybrid Placental Dysplasia in Congenic Mouse Strains.” *Genetics* 153 (1): 383–90.

Hemberger, M C, U Zechner, Wolfgang Mayer, Ralf Krause, Matthais Reule, Bernhard Korn, Rosemary W Elliott, Annemarie Poustka, and Reinald H Fundele. 1998. “Genetic Analysis of Placental Dysplasia in Mouse Interspecific Hybrids.” *Genetical Research* 72 (01): 59–72.

Higashimoto, K, H Soejima, T Saito, K Okumura, and T Mukai. 2006. “Imprinting Disruption of the *Cdkn1c/Kcnq1ot1* Domain: the Molecular Mechanisms Causing Beckwith-Wiedemann Syndrome and Cancer.” *Cytogenetic and Genome Research* 113: 306–12.

Huang, Da Wei, Brad T Sherman, and Richard A Lempicki. 2009a. “Systematic and Integrative Analysis of Large Gene Lists Using DAVID Bioinformatics Resources.” *Nature Protocols* 4 (1): 44–57. doi:10.1038/nprot.2008.211.

Huang, Da Wei, Brad T Sherman, and Richard A Lempicki. 2009b. “Bioinformatics



- Enrichment Tools: Paths Toward the Comprehensive Functional Analysis of Large Gene Lists." *Nucleic Acids Research* 37 (1): 1–13. doi:10.1093/nar/gkn923.
- Ishishita, Satoshi, Kazuma Tsuboi, Namiko Ohishi, Kimiyuki Tsuchiya, and Yoichi Matsuda. 2015. "Abnormal pairing of X and Y sex chromosomes during meiosis I in interspecific hybrids of *Phodopus campbelli* and *P. Sungorus*." *Scientific Reports* 5: 9435–39. doi:10.1038/srep09435.
- Kurz, H, U Zechner, Annie Orth, and Reinald H Fundele. 1999. "Lack of Correlation Between Placenta and Offspring Size in Mouse Interspecific Crosses." *Anatomy and Embryology* 200 (3): 335–43. doi:10.1007/s004290050284.
- Leiser, R, and P Kaufmann. 1994. "Placental Structure: in a Comparative Aspect." *Experimental and Clinical Endocrinology* 102 (3): 122–34. doi:10.1055/s-0029-1211275.
- Li, H, and R Durbin. 2009. "Fast and Accurate Short Read Alignment with Burrows-Wheeler Transform." *Bioinformatics* 25 (14): 1754–60. doi:10.1093/bioinformatics/btp324.
- Li, L L, E B Keverne, S A Aparicio, F Ishino, and S C Barton. 1999. "Regulation of Maternal Behavior and Offspring Growth by Paternally Expressed *Peg3*." *Science* 284: 330–33.
- Liao, Yang, Gordon K Smyth, and Wei Shi. 2014. "featureCounts: an Efficient General Purpose Program for Assigning Sequence Reads to Genomic Features." *Bioinformatics* 30 (7): 923–30. doi:10.1093/bioinformatics/btt656.
- Lim, Derek Hock Kiat, and Eamonn Richard Maher. 2010. "Genomic Imprinting Syndromes

- and Cancer.” *Advances in Genetics* 70: 145–75. doi:10.1016/B978-0-12-380866-0.60006-X.
- Livingstone, K D, G Churchill, and M K Jahn. 2000. “Linkage Mapping in Populations with Karyotypic Rearrangements.” *The Journal of Heredity* 91 (6): 423–28.
- Loschiavo, Maria, Quang K Nguyen, Amanda R Duselis, and Paul B Vrana. 2007. “Mapping and identification of candidate loci responsible for *Peromyscus* hybrid overgrowth.” *Mammalian Genome* 18 (1): 75–85. doi:10.1007/s00335-006-0083-x.
- Martin, Marcel. 2011. “Cutadapt removes adapter sequences from high-throughput sequencing teads.” *EMBnet.Journal* 17: 1–3.
- McCarthy, D J, Y Chen, and G K Smyth. 2012. “Differential Expression Analysis of Multifactor RNA-Seq Experiments with Respect to Biological Variation.” *Nucleic Acids Research* 40 (10): 4288–97. doi:10.1093/nar/gks042.
- McKenna, A, M Hanna, E Banks, A Sivachenko, K Cibulskis, A Kernytsky, K Garimella, et al. 2010. “The Genome Analysis Toolkit: a MapReduce Framework for Analyzing Next-Generation DNA Sequencing Data.” *Genome Research* 20 (9): 1297–1303. doi:10.1101/gr.107524.110.
- Oh-McGinnis, Rosemary, Aaron B Bogutz, and Louis Lefebvre. 2011. “Partial Loss of Ascl2 Function Affects All Three Layers of the Mature Placenta and Causes Intrauterine Growth Restriction” 351 (2): 277–86. doi:10.1016/j.ydbio.2011.01.008.
- Peterson, Brant K, Jesse N Weber, Emily H Kay, Heidi S Fisher, and Hopi E Hoekstra. 2012.

- “Double Digest RADseq: an Inexpensive Method for De Novo SNP Discovery and Genotyping in Model and Non-Model Species.” *PLoS ONE* 7 (5): e37135.  
doi:10.1371/journal.pone.0037135.t001.
- Presgraves, Daven C. 2008. “Sex Chromosomes and Speciation in *Drosophila*.” *Trends in Genetics* 24 (7): 336–43. doi:10.1016/j.tig.2008.04.007.
- Presgraves, Daven C. 2010. “The Molecular Evolutionary Basis of Species Formation.” *Nature Reviews Genetics* 11 (3): 175–80. doi:10.1038/nrg2718.
- Rauscher, FJD. 1993. “The WT1 Wilms Tumor Gene Product: a Developmentally Regulated Transcription Factor in the Kidney That Functions as a Tumor Suppressor.” *The FASEB Journal*. 7(10): 896-903.
- Robinson, M D, D J McCarthy, and G K Smyth. 2010. “edgeR: a Bioconductor Package for Differential Expression Analysis of Digital Gene Expression Data.” *Bioinformatics* 26 (1): 139–40. doi:10.1093/bioinformatics/btp616.
- Robinson, Mark D, and Alicia Oshlack. 2010. “A Scaling Normalization Method for Differential Expression Analysis of RNA-Seq Data” *Genome Biology* 11 (3): R25.  
doi:10.1186/gb-2010-11-3-r25.
- Rodrigue, Sébastien, Arne C Materna, Sonia C Timberlake, Matthew C Blackburn, Rex R Malmstrom, Eric J Alm, and Sallie W Chisholm. 2010. “Unlocking Short Read Sequencing for Metagenomics.” *PLoS ONE* 5 (7): e11840.  
doi:10.1371/journal.pone.0011840.s001.

- Romanenko, Svetlana A, Vitaly T Volobouev, Polina L Perelman, Vladimir S Lebedev, Natalya A Serdukova, Vladimir A Trifonov, Larisa S Biltueva, et al. 2007. "Karyotype Evolution and Phylogenetic Relationships of Hamsters (Cricetidae, Muroidea, Rodentia) Inferred From Chromosomal Painting and Banding Comparison." *Chromosome Research* 15(3): 1–15. doi:10.1007/s10577-007-1124-3.
- Ross, P D. 1995. "*Phodopus Campbelli*." *Mammalian Species* 503. JSTOR: 1–7.
- Safronova, L D, and N Y Vasil'eva. 1996. "Meiotic Abnormalities in Interspecific Hybrids Between *Phodopus Sungorus* (Pallas, 1773) and *Ph. Campbelli* (Thomas, 1905)." *Russian Journal of Genetics* 32 (4): 486–94.
- Safronova, L D, E V Cherepanova, and N Y Vasil'eva. 1999. "Specific Features of the First Meiotic Division in Hamster Hybrids Obtained by Backcrossing *Phodopus Sungorus* And *Phodopus Campbelli*." *Russian Journal of Genetics* 35 (2): 184–88.
- Scribner, S J, and K E Wynne-Edwards. 1994. "Disruption of Body Temperature and Behavior Rhythms During Reproduction in Dwarf Hamsters (*Phodopus*)." *Physiology & Behavior* 55 (2): 361–69.
- Simon-Chazottes, Dominique, and X Montagutelli. 1990. "*Hst-3*: an X-Linked Hybrid Sterility Gene." *Genetical Research* 56: 163–65.
- Sokolov, V E, and N I Vasil'eva. 1993. "Hybridologic Analysis Confirms the Species Specificity of *Phodopus Sungorus* (Pallus, 1773) and *Phodopus Campbelli* (Thomas, 1905)." *Doklady Akademii Nauk* 332 (1): 120–23.

- Surani, M A, and S C Barton. 1983. "Development of Gynogenetic Eggs in the Mouse: Implications for Parthenogenetic Embryos." *Science* 222 (4627): 1034–36.
- Takada, Hisashi, Naoki Wakabayashi, Osamu Dohi, Kohichiroh Yasui, Chouhei Sakakura, Shoji Mitsufuji, Masafumi Taniwaki, and Toshikazu Yoshikawa. 2010. "Tissue Factor Pathway Inhibitor 2 (*Tfpi2*) Is Frequently Silenced by Aberrant Promoter Hypermethylation in Gastric Cancer." *Cancer Genetics and Cytogenetics* 197 (1): 16–24. doi:10.1016/j.cancergencyto.2009.11.004.
- Tao, Yun, Sining Chen, Daniel L Hartl, and Cathy C Laurie. 2003. "Genetic Dissection of Hybrid Incompatibilities Between *Drosophila Simulans* and *D. Mauritiana*. I. Differential Accumulation of Hybrid Male Sterility Effects on the X and Autosomes." *Genetics* 164 (4): 1383–97.
- Theiler, K. 1972. *The House Mouse. Development and Normal Stages From Fertilization to 4 Weeks of Age*. New York, NY: Springer-Verlag.
- Turelli, M, and H Allen Orr. 1995. "The Dominance Theory of Haldane's Rule." *Genetics* 140 (1): 389–402.
- Turelli, M, and H Allen Orr. 2000. "Dominance, Epistasis and the Genetics of Postzygotic Isolation." *Genetics* 154 (4): 1663–79.
- Turelli, Michael, and Leonie C Moyle. 2007. "Asymmetric Postmating Isolation: Darwin'S Corollary to Haldane'S Rule." *Genetics* 176 (2): 1059–88. doi:10.1534/genetics.106.065979.

- Van der Auwera, Geraldine A, Mauricio O Carneiro, Chris Hartl, Ryan Poplin, Guillermo del Angel, Ami Levy-Moonshine, Tadeusz Jordan, et al. 2013. "From FastQ Data to High Confidence Variant Calls: the Genome Analysis Toolkit Best Practices Pipeline." *Current Protocols in Bioinformatics* 11: 11-33. doi:10.1002/0471250953.bi1110s43.
- Van Hoosier, G L, Jr. 1987. "Laboratory Hamsters." American College of Laboratory Animal Medical Series.
- Varmuza, S. 1993. "Gametic Imprinting as a Speciation Mechanism in Mammals." *Journal of Theoretical Biology* 164 (1): 1-13. doi:10.1006/jtbi.1993.1137.
- Vorontsov, N N. 1967. "Karyologic Differentiation of Allopatric Forms of the Hamster Super-Species *Phodopus Sungorus* And the Heteromorphism of Chorosomes in Females." *Doklady Akademii Nauk* 172 (3): 703-5.
- Vrana, P B. 2007. "Genomic Imprinting as a Mechanism of Reproductive Isolation in Mammals." *Journal of Mammalogy* 88 (1): 5-23.
- Vrana, P B, J A Fossella, P Matteson, T del Rio, M J O'Neill, and S M Tilghman. 2000. "Genetic and Epigenetic Incompatibilities Underlie Hybrid Dysgenesis in *Peromyscus*." *Nature Genetics* 25 (1): 120-24. doi:10.1038/75518.
- Vrana, P B, X J Guan, R S Ingram, and S M Tilghman. 1998. "Genomic Imprinting Is Disrupted in Interspecific *Peromyscus* Hybrids." *Nature Genetics* 20 (4): 362-65. doi:10.1038/3833.
- Wake, N, N Takagi, and M Sasaki. 1976. "Non-Random Inactivation of X Chromosome in the

Rat Yolk Sac." *Nature* 262: 580–81.

Wang, Xu, D C Miller, and R Harman. 2013. "Paternally Expressed Genes Predominate in the Placenta." *PNAS* 110(26): 10705–10. doi:10.1073/pnas.1308998110.

Wolf, Jason B, and Y Brandvain. 2014. "Gene Interactions in the Evolution of Genomic Imprinting." *Heredity* 113 (2): 129–37. doi:10.1038/hdy.2014.7.

Zechner, U, M Reulez, Annie Orth, and F Bonhomme. 1996. "An X-Chromosome Linked Locus Contributes to Abnormal Placental Development in Mouse Interspecific Hybrids." *Nature Genetics* 12 (4): 398–403. doi:10.1038/ng0496-398.

Zechner, U, Matthais Reule, P S Burgoyne, A Schubert, Annie Orth, H Hameister, and Reinald H Fundele. 1997. "Paternal Transmission of X-Linked Placental Dysplasia in Mouse Interspecific Hybrids." *Genetics* 146 (4): 1399–1405.

Zechner, U, W Shi, M C Hemberger, H Himmelbauer, Sabine Otto, Annie Orth, V Kalscheuer, et al. 2004. "Divergent Genetic and Epigenetic Post-Zygotic Isolation Mechanisms in Mus and Peromyscus." *Journal of Evolutionary Biology* 17 (2): 453–60. doi:10.1046/j.1420-9101.2003.00656.x.

Figure 1

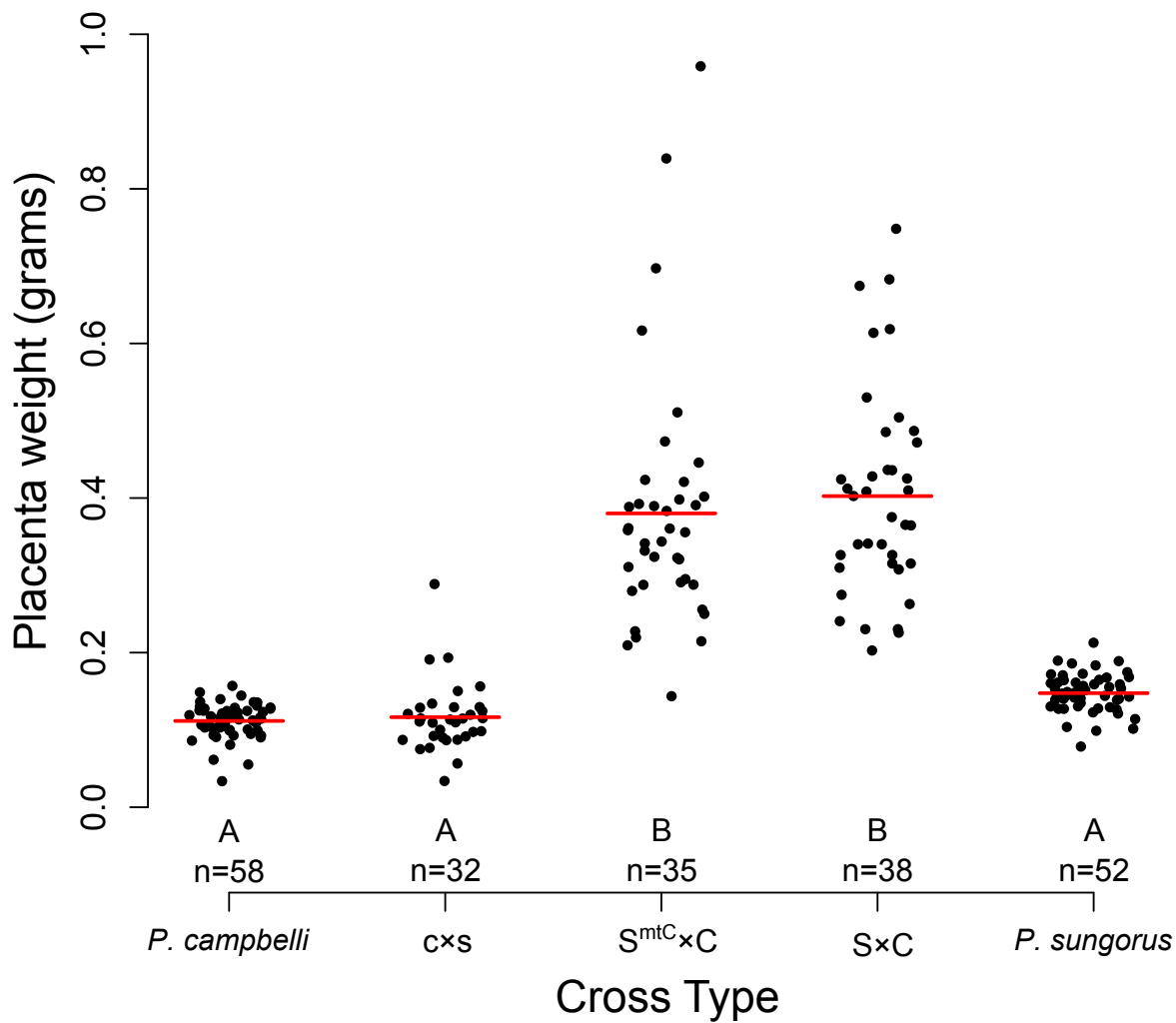




Figure 2

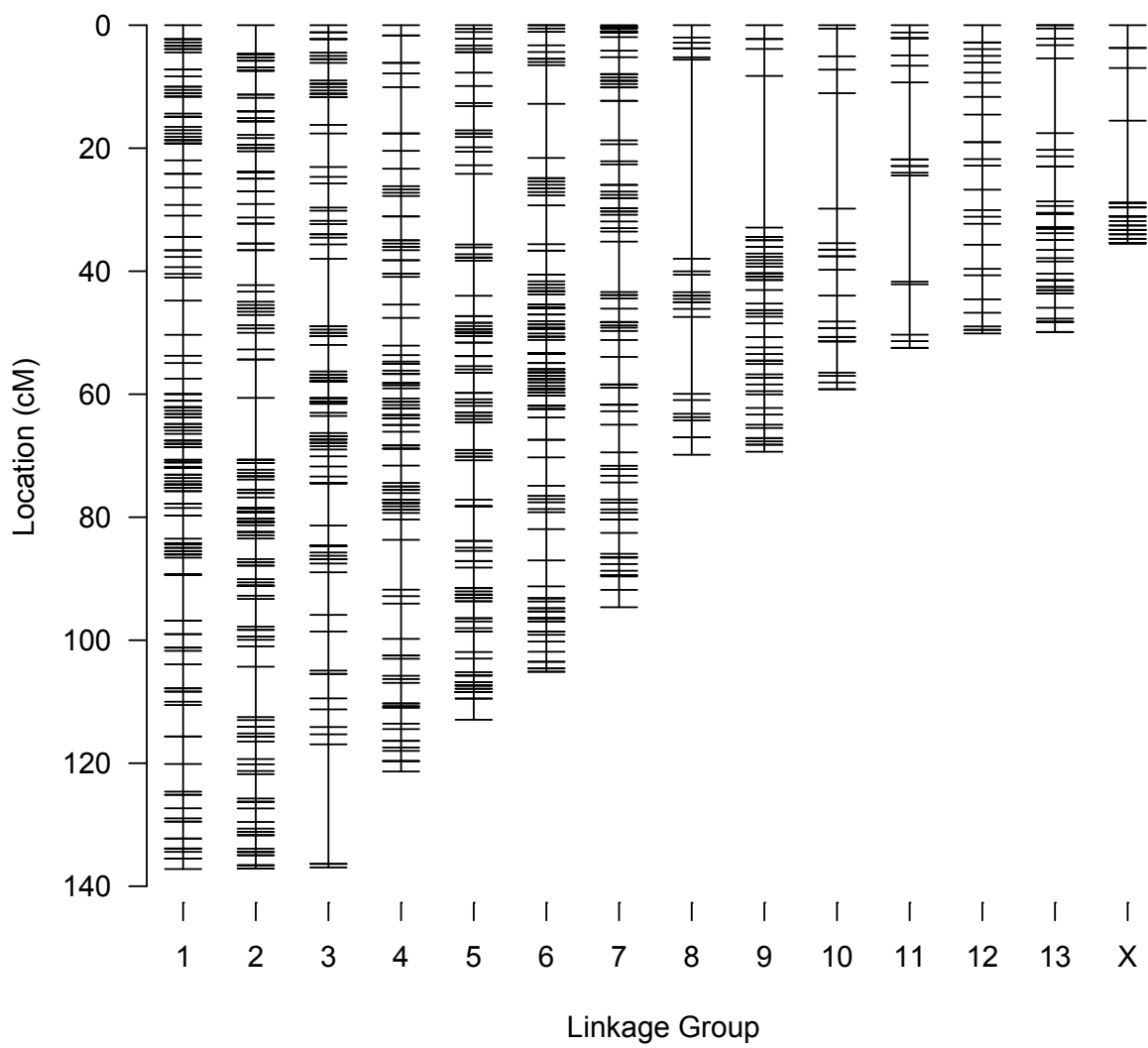


Figure 3

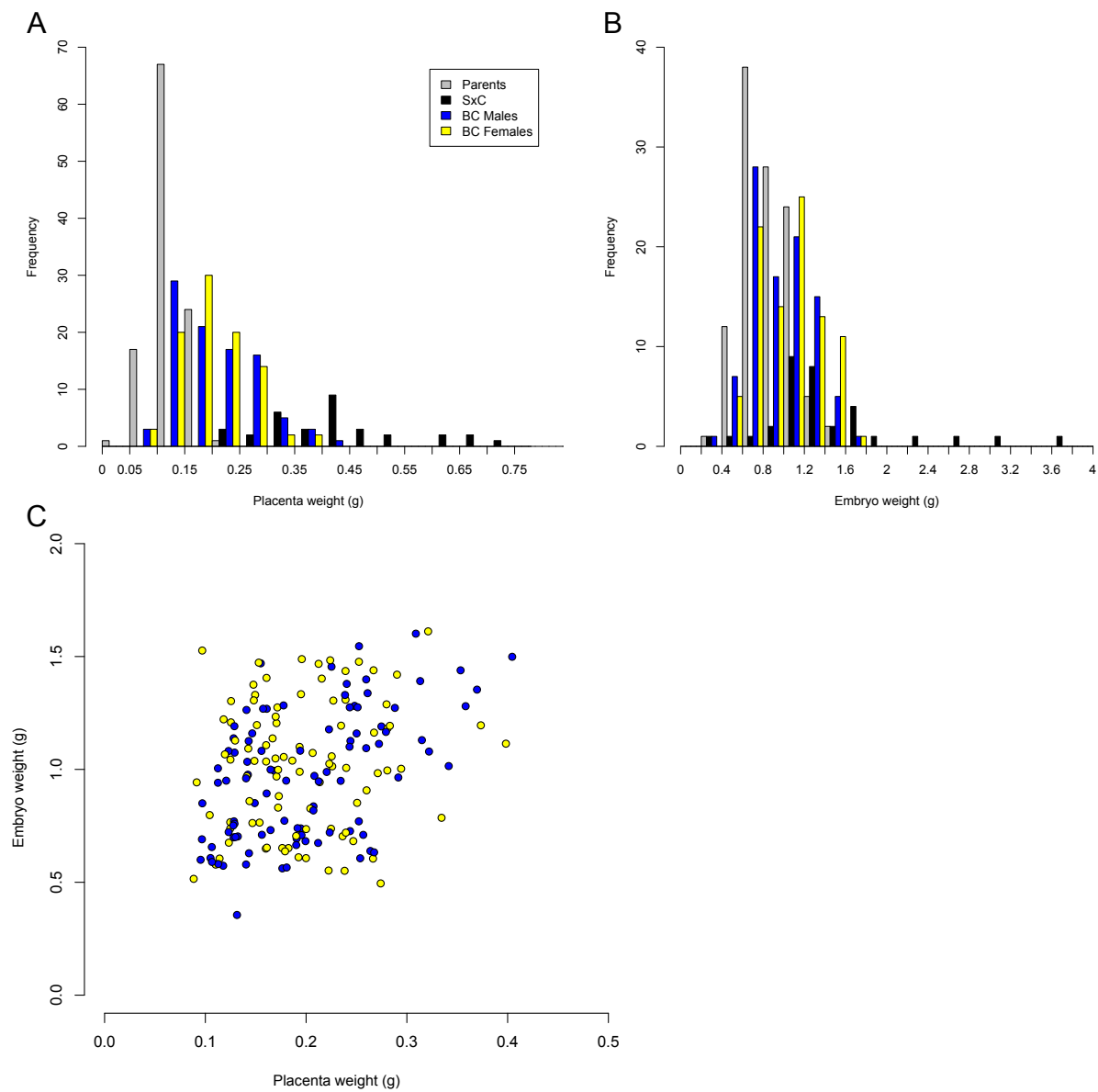


Figure 4A

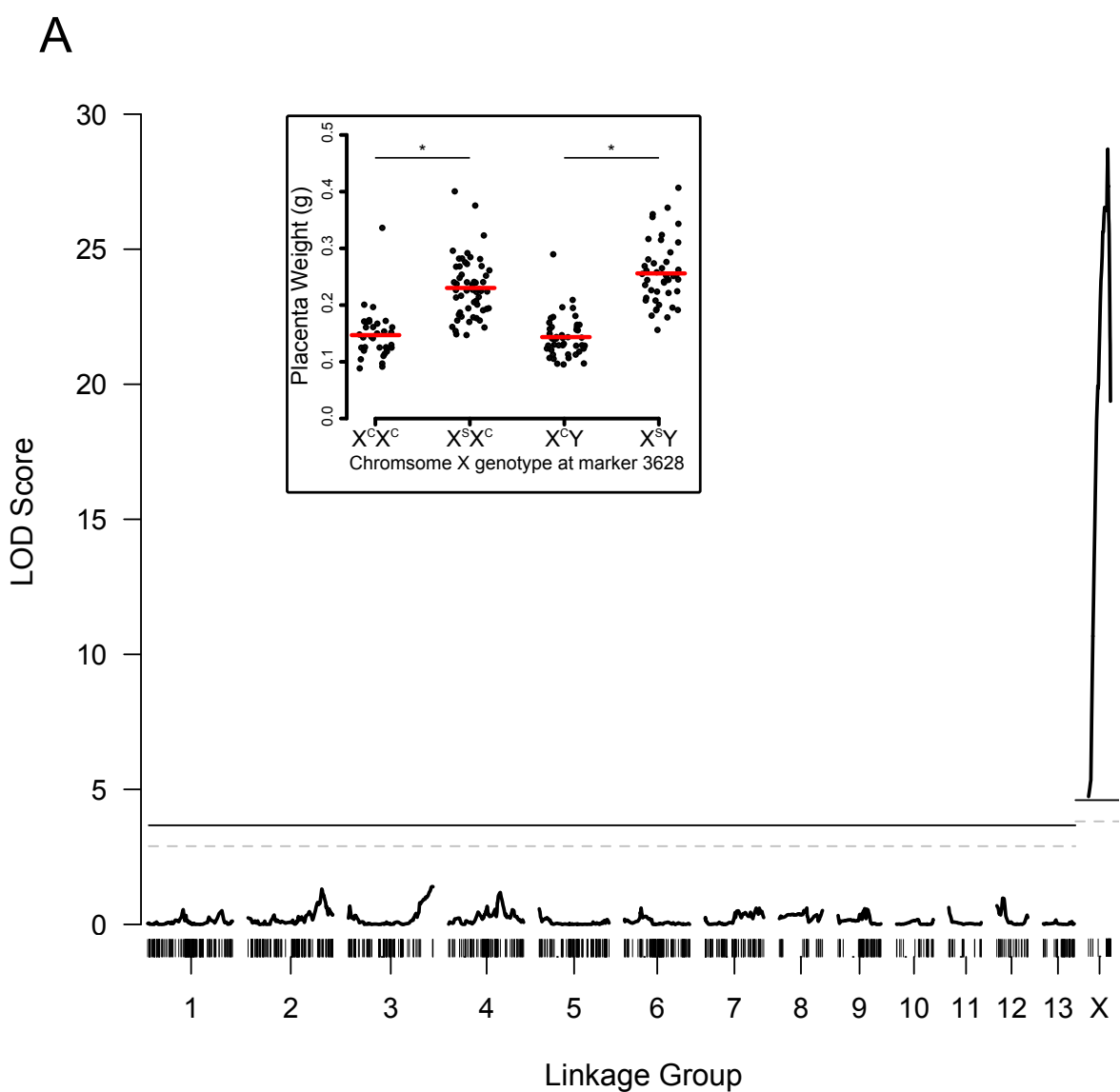


Figure 4B

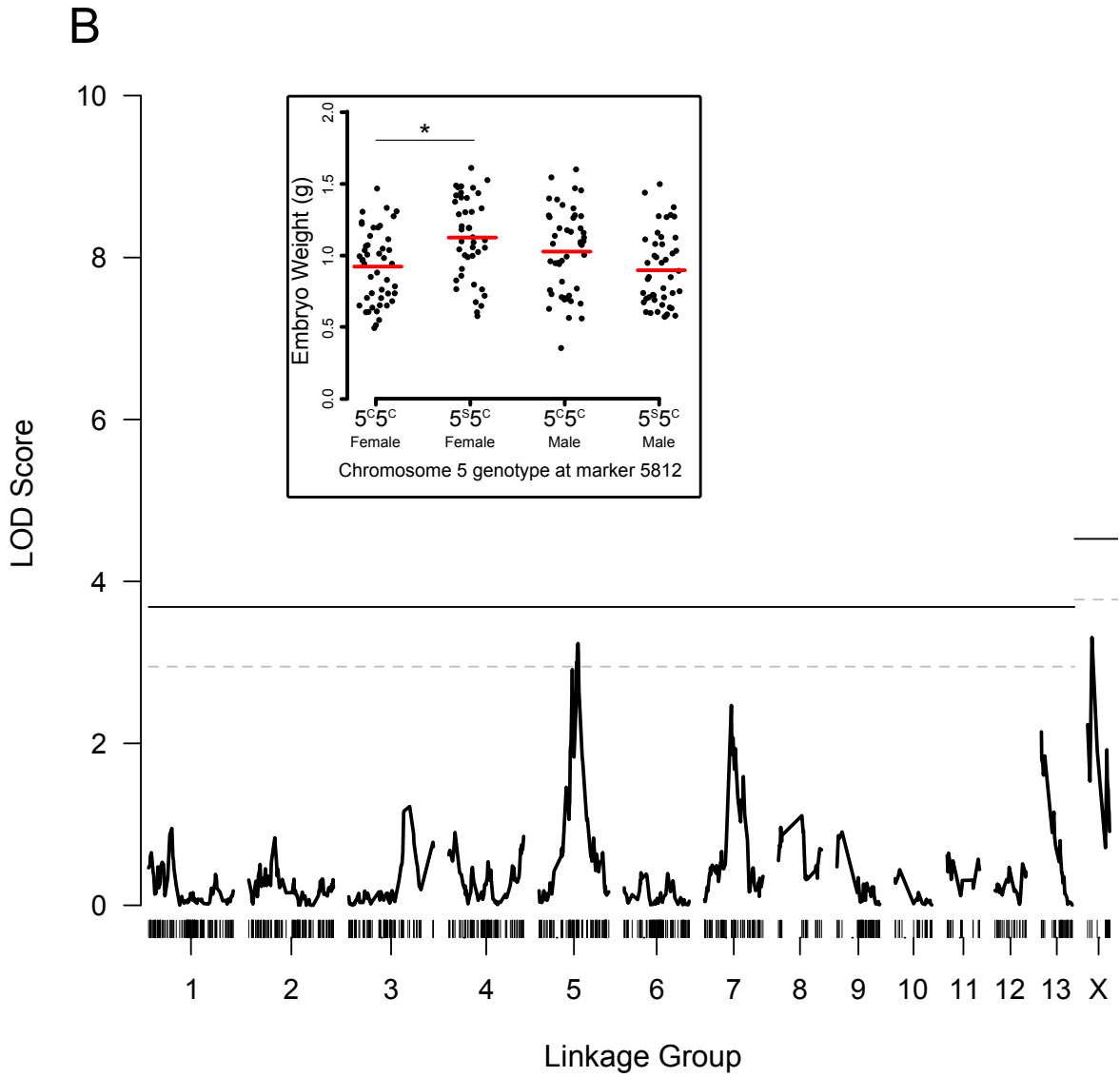


Figure 5

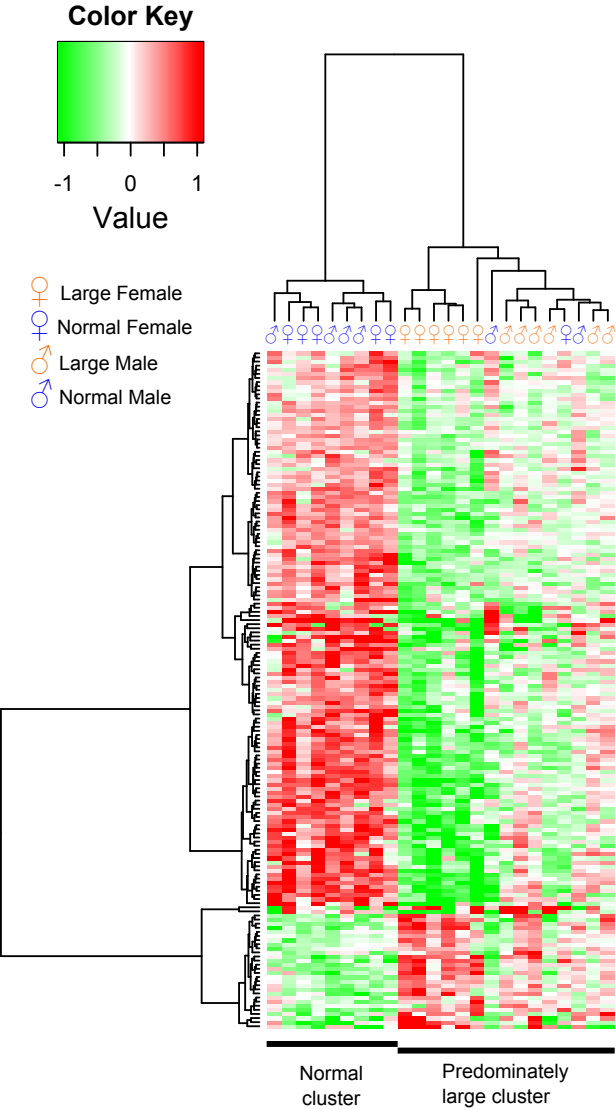
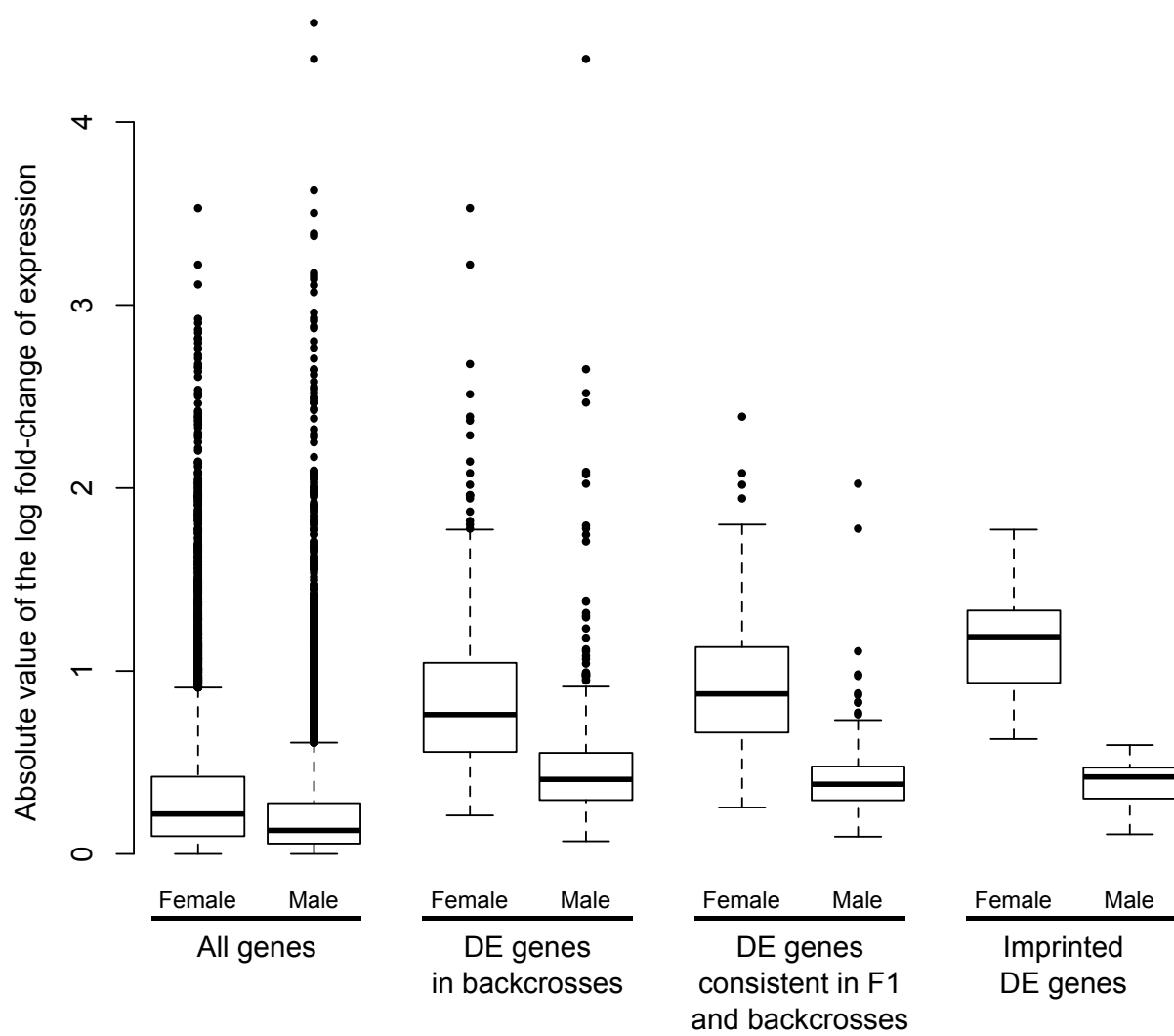


Figure 6



## Supplemental Table 1

This is an enormous table of RAD sequences and meta-data. Accessible at [www.tombrekke/research/STable\\_1\\_RAD\\_database.csv](http://www.tombrekke/research/STable_1_RAD_database.csv)

Supplemental Table 2A: DAVID enrichment for DE genes between large and normal backcrosses

Enrichment Group	Enrichment Score	Summary Term	Enrichment Terms
1	2.41	Vasculature development	angiogenesis blood vessel morphogenesis blood vessel development vasculature development

Supplemental Table 2B: DAVID enrichment for genes with lower expression in large backcrosses than normal backcross:

Enrichment Group	Enrichment Score	Summary Term	Enrichment Terms
1	2.77	Angiogenesis	angiogenesis blood vessel morphogenesis blood vessel development vasculature development enzyme linked receptor protein signaling pathway transmembrane receptor protein tyrosine kinase signaling pathway
2	2.24	Cell migration	cell motion cell migration cell motility localization of cell
3	2.28	Regulation of Locomotion	negative regulation of cell migration negative regulation of locomotion negative regulation of cell motion regulation of cell motion regulation of cell migration regulation of locomotion